A MORE CONVENIENT ROUTE TO LABELED LIPOIC ACID

by

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To

my wife

and

parents

ACKNOWLEDGEMENT

I wish to express my deepest appreciation for the guidance and encouragement given me by Dr. Joseph V. Paukstellis, without whose help this work would not have been completed.

My personal thanks go to Dr. William G. Fateley for all the help he provided me so generously.

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INTRODUCTION

Lipoic acid is a prosthetic group of the enzyme system for the oxidation of pyruvate and others α-keto acids (e.g. α-ketoglutarate, α-ketoisovalerate, α-ketoisocaproate, α-keto-β-methylvalerate, glyoxalate, etc..). It has been proposed that lipoic acic functions in acyl transfer to Coenzyme A and electron transfer to flavine adenine dinucleotide.[1,2]

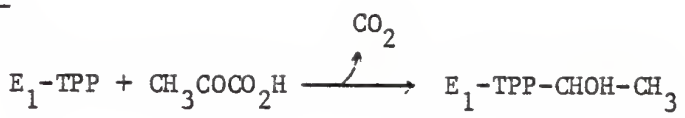
The oxidation of pyruvate to acetyl-CoA illustrates the general role of lipoic acid in these oxidations. The overall equation is;

$$\text{pyruvate} + NAD^+ + CoA \longrightarrow \text{acetylCoA} + NADH + H^+ + CO_2 \ .$$

This reaction is reversible in animal tissues ($\Delta G^{\circ\prime} = -8.0$ kcal mol^{-1}) and is necessary for the entry of all carbohydrates into the tricarboxylic acid cycle. The pyruvate dehydrogenase complex, a multienzyme complex of three different enzymes and five different coenzymes, catalyzes the reaction. The five reaction steps promoted by this complex are illustrated in Figure 1.

Pyruvate dehydrogenase (E_1), whose prosthetic group is the coenzyme thiamin pyrophosphate (TPP), is involved in step I. Pyruvate undergoes enzymatically decarboxylation to yield carbon dioxide (CO_2) and the α-hydroxyethyl derivative of TPP (HETPP). In step II the hydroxyl group is dehydrogenated to an acetyl group, which is transferred to the sulfur atom at carbon 6 or 8 of lipoic acid. The lipoic acid is a covalently bound prosthetic group of the second enzyme complex, lipoate acetyl transferase (E_2).

STEP I



STEP II

STEP III

STEP IV

STEP V

Figure 1. Steps in the oxidation of pyruvate to acetyl-CoA by the pyruvate dehydrogenase complex.

Lipoic acid is attached by an amide linkage to the α-amino group of a lysine residue in the enzyme complex (Figure 2). The transfer of a pair of electrons from the hydroxyethyl group of thiamine pyrophosphate to the disulfide bond of lipoic acid converts the latter to the correspondent reduced, dithiol form, dihydrolipoic acid. In step III the acetyl group is enzymatically transferred from the lipoyl group of acetyldihydrolipoic acid to the thiol group of coenzyme A. The acetyl-CoA, thus formed, leaves the enzyme complex in free form. In step IV the dithiol form of the lipoyl group is reoxidized to the disulfide form by the third enzyme of the complex, lipoamide dehydrogenase (E_3). The reducible prosthetic group of E_3 is tightly bound flavin adenine dinucleotide (FAD). The resulting $FADH_2$ remains bound to the enzyme and is reoxidized in step V by NAD^+, with formation of NADH.

Figure 3 illustrates that the mobility of the long lipoyllysyl side chain of E_2 is an important feature in understanding the role of lipoic acid in the pyruvate dehydrogenase complex . the lipoyllysyl is free to swing from site to site and undergoes the various reactions. In the disulfide form the chain swings to the TPP site of E_1 and undergoes a transacetylation to the dihydro form. The side chain then moves to the active site of E_2 where it transfers the acetyl group to CoA. Now in its reduced form, it swings to the FAD site of E_3 and is converted to the oxidized form. The oxidized disulfide form can now return to the TPP site of E_1 and repeat the cycle.

The question that remains to be answered concerning this

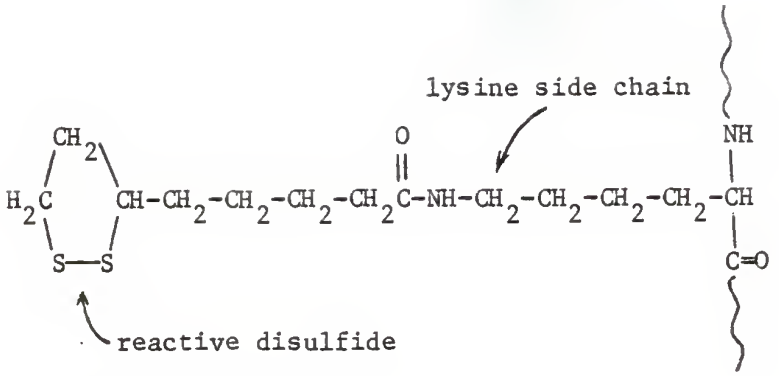


Figure 2. Lipoamide.

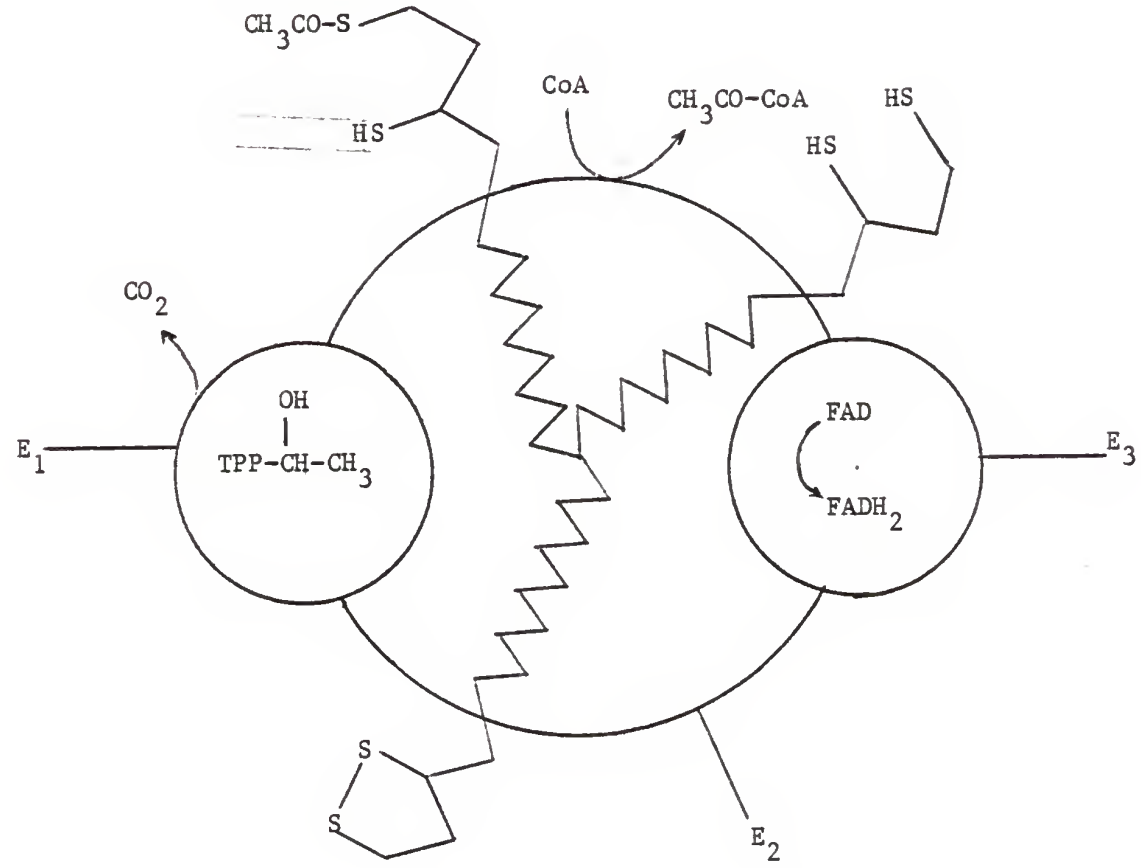


Figure 3. Role of lipoyllysyl group in the pyruvate dehydrogenase complex.

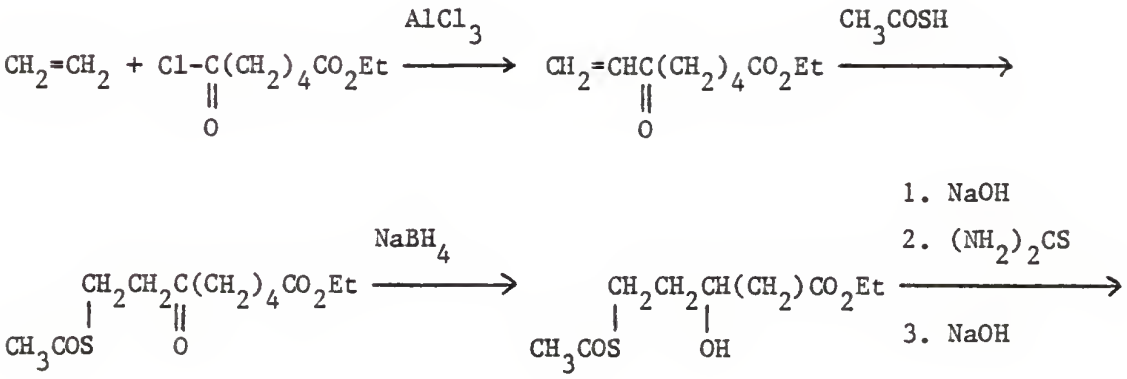
"swinging arm" mechanism is whether the acetyl group obtained from TPP is attached to the 6 or 8 position of lipoic acid . Gunsalus and coworkers[3] reported that the acetyl group is attached to the 6 position. The evidence for this conclusion was the reaction of their product with N-propionyldiphenylketimine. Since N-propionyldiphenylketimine is apparently specific for primary -SH groups, it was concluded that the enzymatic reaction results in acetylation of the secondary -SH group. Reed[4] believed that it should be kept in mind that Gunsalus' enzymatic transacetylation reactions were carried out under nonphysiological conditions involving nonbound lipoic acid. It should not necessary follow, therefore, that these model reactions mirror transformation of the bound lipoic acid.

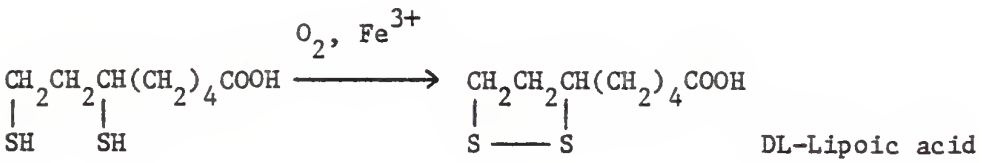
It may be possible to solve this problem by using NMR and/or radioactive detection techniques to study the role of lipoic acid in the transacetylation reactions catalyzed by these multienzyme complexes. For instance, in the study of the pyruvate dehydrogenase complex, it may be feasible to determine whether the acetyl group is attached specifically to the 6 or 8 position of lipoic acid, randomly to the 6 or 8 positions, or intramolecularly transfers between the two positions.

This thesis does not attempt to solve this problem. It does however present the ground work necessary to a study of this nature by providing a convenient route to labeled lipoic acid.

The synthesis of lipoic acid developed by Bullock[14,15] which unequivocally established the structure of lipoic acid, gave DL-lipoic acid in an overall yield of approximately 14% starting

from ethyl adipyl chloride.

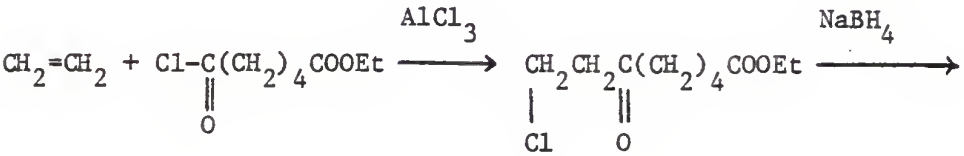


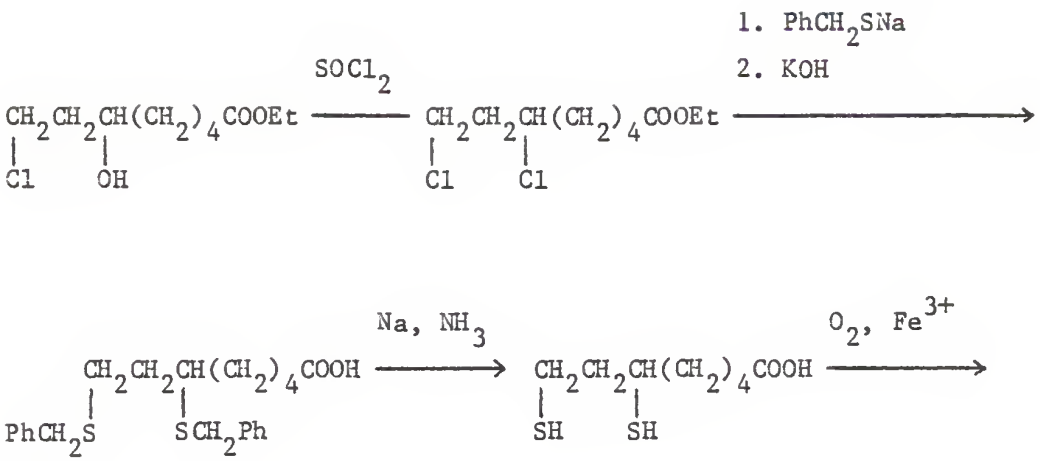


DL-Lipoic acid

Modifications which improved this synthesis involved the direct conversion of structurally related substances to 6,8-dithioloctanoic acid by reduction in the presence of H_2, H_2S and a cobalt sulfide catalyst.[16,17] Yields of crystalline DL-lipoic acid of from 30 to 39% have been obtained by this procedure starting from methyl-6-oxo-7-octenoate.

Reed and Niu[18] described a synthesis which gave DL-lipoic acid in 36% yield based on the intermediate ethyl adipyl chloride.





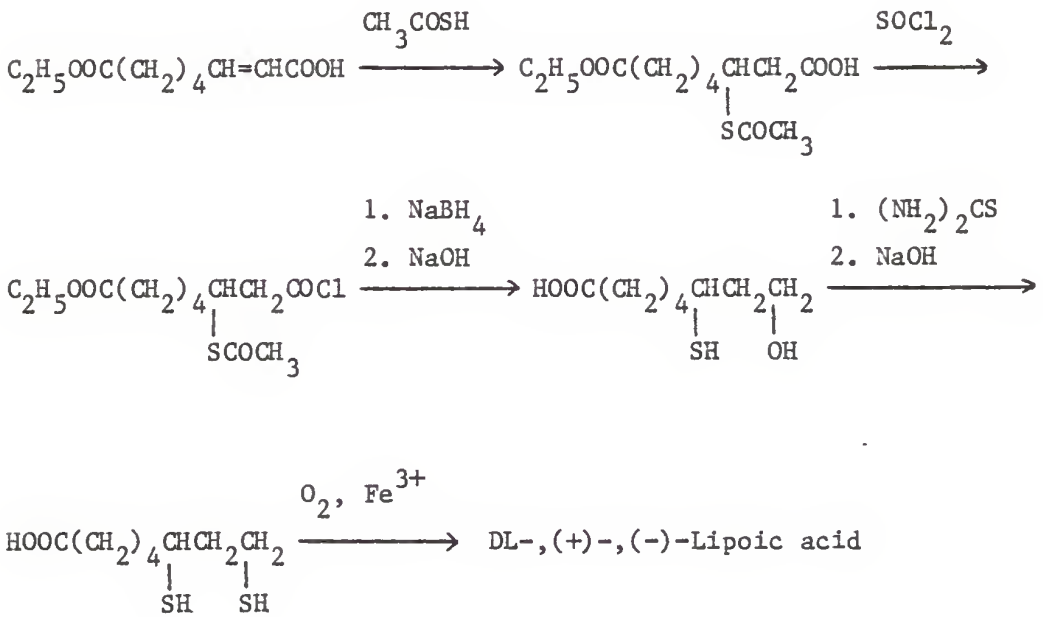
DL-Lipoic acid

By suitable modification of this synthesis DL-lipoic acid-S^{35} of high specific activity has been made available for biological experiments.[19,20]

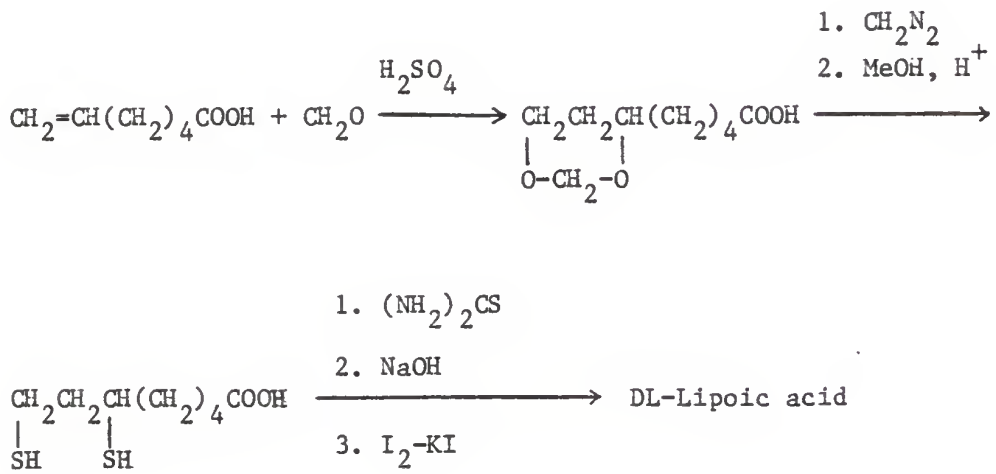
6,8-Dichlorooctanoic acid and its esters were converted to DL-lipoic acid in yield as high as 68% by reaction of former substances with sodium disulfide.[21]

The 6,8-dichlorooctanoic acid was also resolved with ephedrine, thereby providing convenient route to (+)-lipoic acid and its optical antipode. Sulfur-S^{35} has been used in this synthesis to prepare S^{35}-labeled DL-lipoic acid

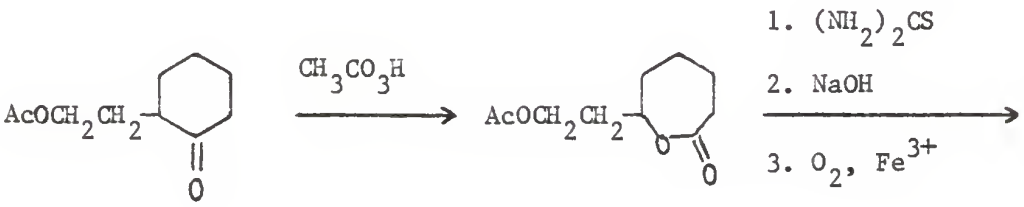
A synthesis developed by Walton et al.[22] gave an overall yield of 21% of (+)-,(-)-lipoic acid from 7-carbethoxy-2-heptenoic acid.



From 6-heptenoic acid, DL-lipoic acid was prepared in 32% yield by the reaction sequence shown below.[23]

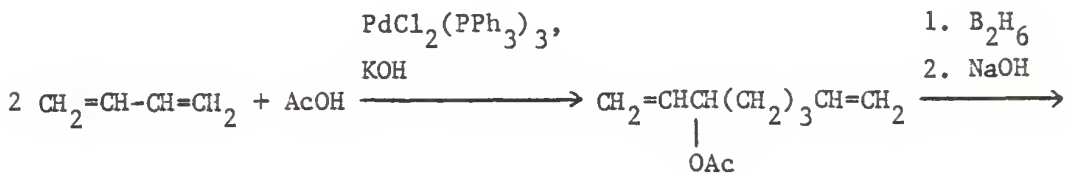


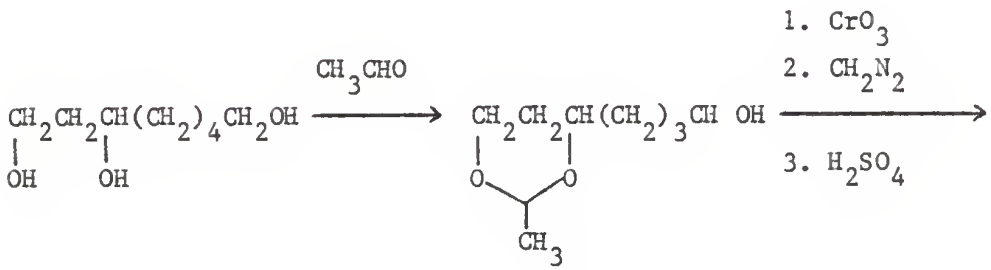
Segre et al.[6] obtained DL-lipoic acid from 2-acetoxyethylcyclohexanone by the reaction sequence shown below. The overall yield was 20.4% from cyclohexanone.

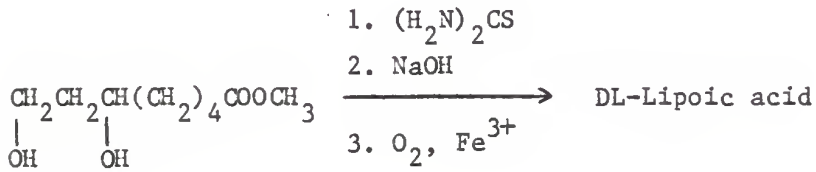


DL-Lipoic acid

Recently, a new synthetic method for DL-lipoic acid has been reported, using a butadiene telomer as a starting material. The yield was 13.2% from 3-acetoxy-1,7-octadiene.[24]







OBJECTIVE OF THIS INVESTIGATION

The objective of this investigation is to develop a more convenient route that could be used for the synthesis of ^{13}C, ^{2}H, ^{3}H and ^{35}S-labeled lipoic acid.

DISCUSSION OF EXPERIMENTAL RESULTS

The need for labeling lipoic acid at either C_6, C_7 or C_8 with ^{13}C, ^{2}H, ^{3}H or ^{35}S required that a sequence be developed that would allow specific labeling at any or all the sites. The initial approach required only 2 or 3 isolations and is given below in Fig. 4.

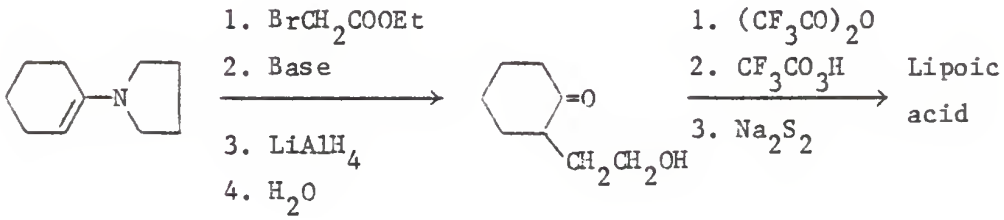


Figure 4. Initial approach of lipoic acid synthesis.

In addition to the fewer isolations required, the sequence allowed the ^{13}C or ^{14}C labeling of lipoic acid at C_7 or C_8 by using appropriately labeled ethyl bromoacetate, the ^{2}H or ^{3}H labeling at C_8 by reduction with appropriate lithium aluminum hydride reagents or the ^{35}S labeling at C_6 or/and C_8 by using labeled thiourea or sodium disulfide. The first sequence of steps leading to 2-(2-hydroxyethyl)cyclohexanone proceeded without incident and is described in Fig. 5 and in the following discussion.

The enamine was first discovered by Mannich and Davidsen[40] from reaction between aldehyde and secondary amine in the presence of potassium carbonate. In the case of ketones calcium

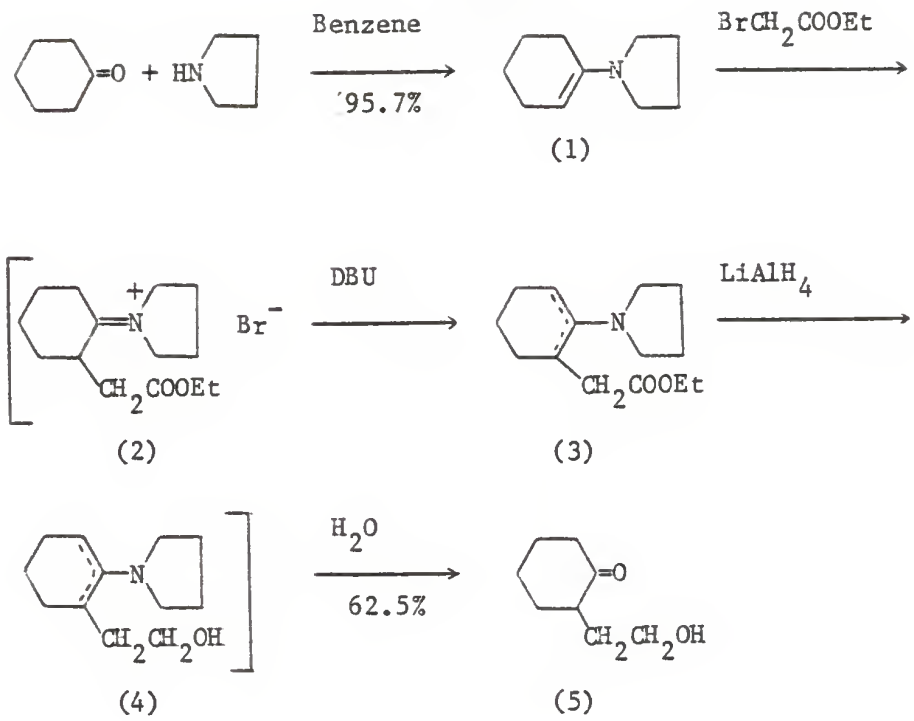


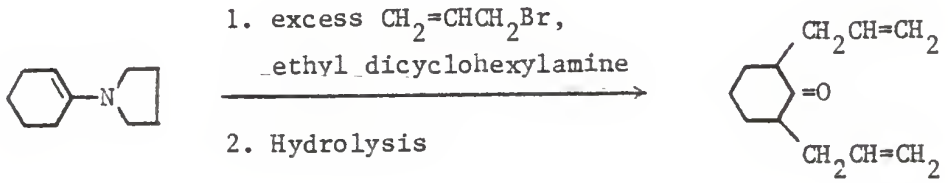
Figure 5. Part I of lipoic acid synthesis.

hydroxide was used and higher temperature were required, and the reaction appeared to be more limited in scope.

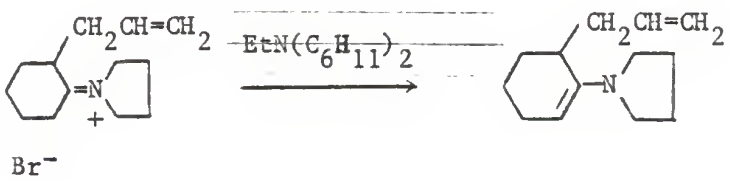
A more useful procedure for the preparation of enamines was developed by Herr and Heyl.[41,42] A solution of the carbonyl compound and an excess cyclic secondary amine in benzene is refluxed under nitrogen and the water removed azeotropically by means of a water separator. Addition of a catalytic amount of p-toluenesulfonic acid might be needed to accelerate the reaction in case of substituted ketones.[43]

From following Herr's method, we have prepared 1-pyrrolidinocyclohexene (1) in excellent yield (95.7%).

The enamine could be alkylated with ethyl bromoacetate to give the intermediate alkylated iminium bromide (2). The advantage of this enamine alkylation reaction over direct alkylation of cyclohexanone under influence of a strong base is that the major product is the monoalkylated derivative.[44,45] When dialkylation is observed, it occurs at the least substituted carbon in contrast to alkylation with base, where the α-substituted product is formed. Dialkylation becomes a predominant reaction when a strong organic base is added and an excess alkyl halide is used.[44]



The course of the reaction appears to involve dehydrohalogenation of the intermediate iminium salt to the new enamine, which then undergoes further alkylation.



In our case, we have treated the intermediate alkylated iminium bromide (2) with 1,5-diazabicyclo [5.4.0] undec-5-ene (DBU) in a 1:1 molar ratio to give 99.9% of DBU hydrobromide

and the corresponding alkylated enamine (3). The high yield and purity of DBU hydrobromide by-product suggested that the conversion was completed successfully. It might be expected that the bulky quinuclidine, DBN, EDCA ... would also effect the conversion since the ideal reagent for such a reaction would be a strong base and also be a poor nucleophyle.

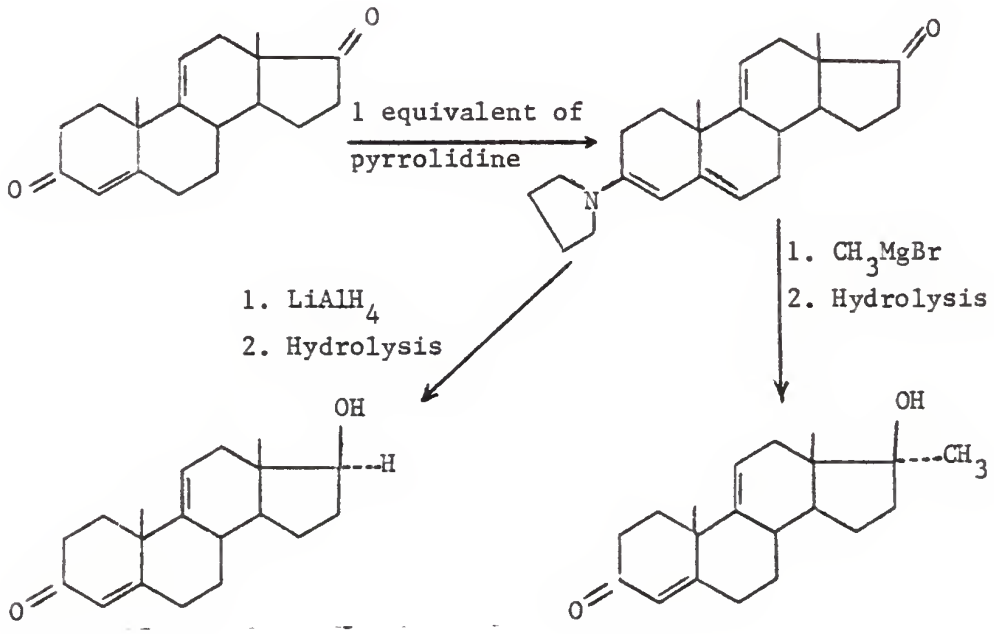
We need to point out that the dehydrobromination of iminium bromide is not easily achieved since the carbon center of $C=N^+$ bond is subjected to nucleophilic attack.[46]

where MX could be lithium aluminum hydride[31], sodium borohydride[32], potassium cyanide, alkyl magnesium halide, alkyl lithium, potassium sulfide[33], diazomethane and even alcohol in some particular cases[35].

The conversion of iminium to enamine was, in fact, unsuccessful with the use of sodium methoxide, tert-butoxide, pyridine, pyrrolidine and triethylamine.

We have made use of the stability of enamines to reduction with lithium aluminum hydride to protect the keto group since in free base form enamines do not react with anions in a non-polar solvent like ether, benzene or tetrahydrofuran. If one is dealing with a polyfunctional molecule containing carbonyl

groups it is possible, by proper choice of a secondary amine, to protect one of these groups while the nucleophilic attack is carried out on the others which are not protected.[41,47,48]



In our case the enamine-ester (3) was reduced with lithium aluminum hydride to give the enamine-alcohol (4) which was easily hydrolyzed in aqueous methanol to yield 62.5% of 2-(2-hydroxyethyl)-cyclohexanone (5).

With the successful preparation of (5) we proceeded with the planned synthesis. We tried to esterify and oxidized the keto-alcohol (5) with trifluoroacetic anhydride and trifluoroperacetic acid in the same reaction flask with the hope of obtaining 6,8-bis-trifluoroacetoxyoctanoic acid which

should be easily converted into lipoic acid, however more than ten attempts under a variety of conditions failed to give the desired products. Upon isolating the intermediate products, only intractable are generally found and could not be identified. We concluded that the first step, the reaction of trifluoroacetic anhydride, was the culprit and that further attempts along the line would be fruitless. We attempted to further shorten the synthetic route of lipoic acid as outline in Figure 6 however the attempts were also unsuccessful. We tried a one-step preparation of (5) by reacting the enamine (1) with bromoethanol; only a water-soluble polymeric product was resulted and this was probably ethylene glycol polymer. Another attempted alkylation of the enamine with 2-bromoethyl acetate failed. The failure to significantly shorten the lipoic acid synthesis forced us to reconsider the synthesis reported by Segre.[6] This synthesis shown early on p.9 in the introduction of this thesis, gave an overall yield of 20.4% starting from cyclohexanone. The first half of the synthesis was based on the same reaction, ie., the alkylation of the enamine, but the basic approach was substantially different. The second half of the synthesis was adapted directly but with some modifications to improve yields. From Segre's approach [6], the keto-alcohol (5) was acetylated to give 94% of 2-(2-oxocyclohexyl)-ethyl acetate (6) which readily undergoes the Baeyer-Villiger[36] oxidation. This type of reaction, the conversion of aliphatic and cyclic ketones into the corresponding esters and lactones,

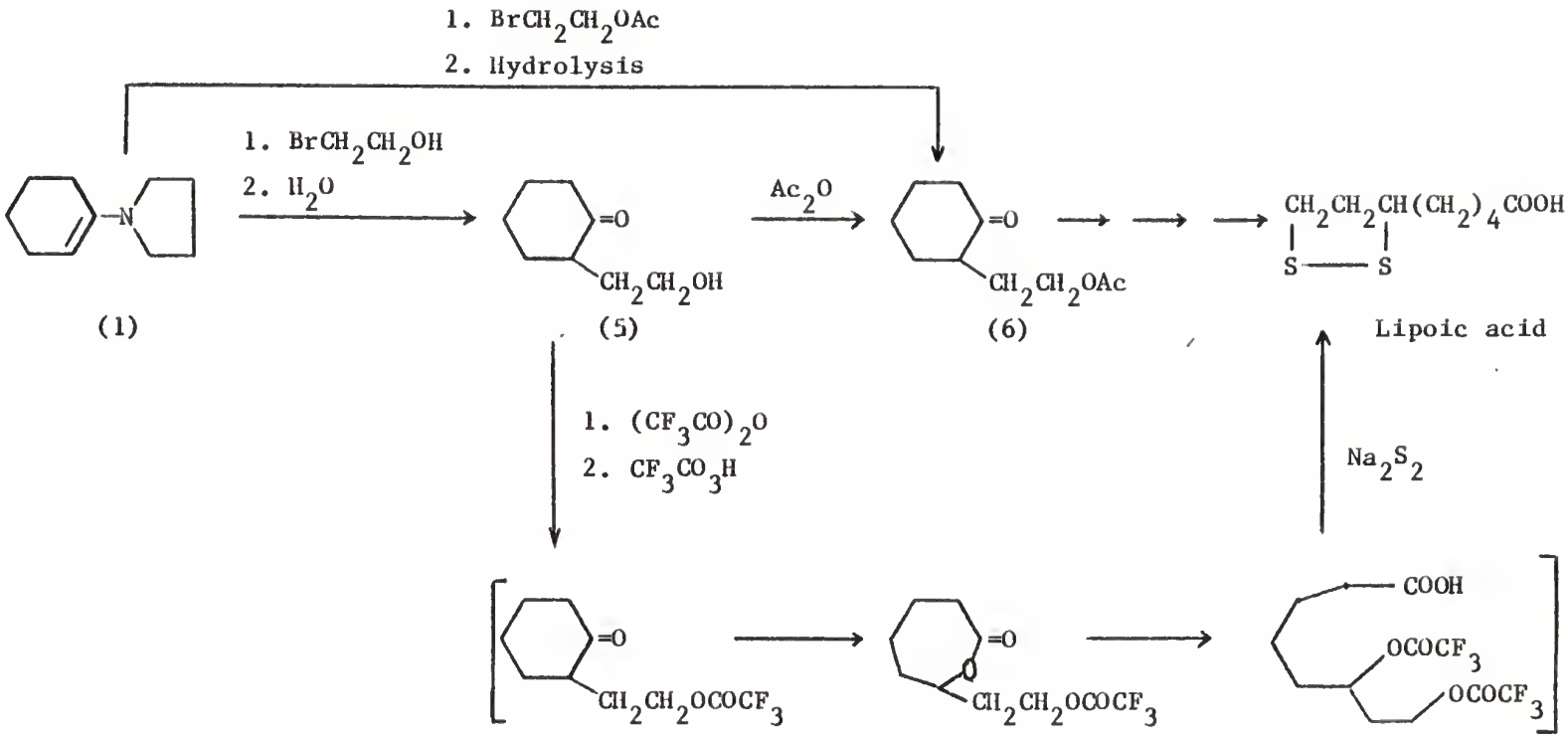
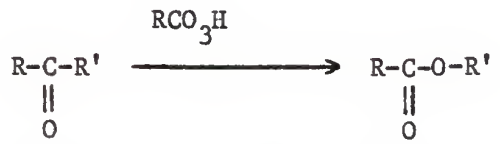


Figure 6. Possible routes leading to the preparation of lipoic acid.

first described by Baeyer and Villiger, has been carried out with peracetic, perbenzoic and Caro's acid.[37] It has been



where R' is a larger group

generally applied to aliphatic, cyclic and aromatic ketones, however, the yields in most cases are not extremely good, and this makes the separation of the ester product from the original ketone somewhat tedious since the materials normally have similar boiling points. The powerful trifluoroperacetic acid developed by Emmons[38], in contrast to other peracids, smoothly and rapidly converts most ketones into esters in very good yields. In addition, the products obtained from this oxidation reaction are in general uncontaminated by any of the ketonic starting material. It was observed that transeterification of the ester by trifluoroacetic acid produced a small amount of alkyl trifluoroacetate. This was eliminated, however by carrying out the oxidation in the presence of disodium phosphate, since this base removed most of trifluoroacetic acid as soon as it is formed. Furthermore, trifluoroperacetic acid has been applied successfully to the oxidation of cyclopentanone and cyclohexanone.[39]

Using the procedure developed by Emmons[38], we carried out the oxidation of 2-(2-oxocyclohexyl)ethyl acetate (6) and obtained 75% of the expected 6-hydroxy-8-acetoxyoctanoic acid

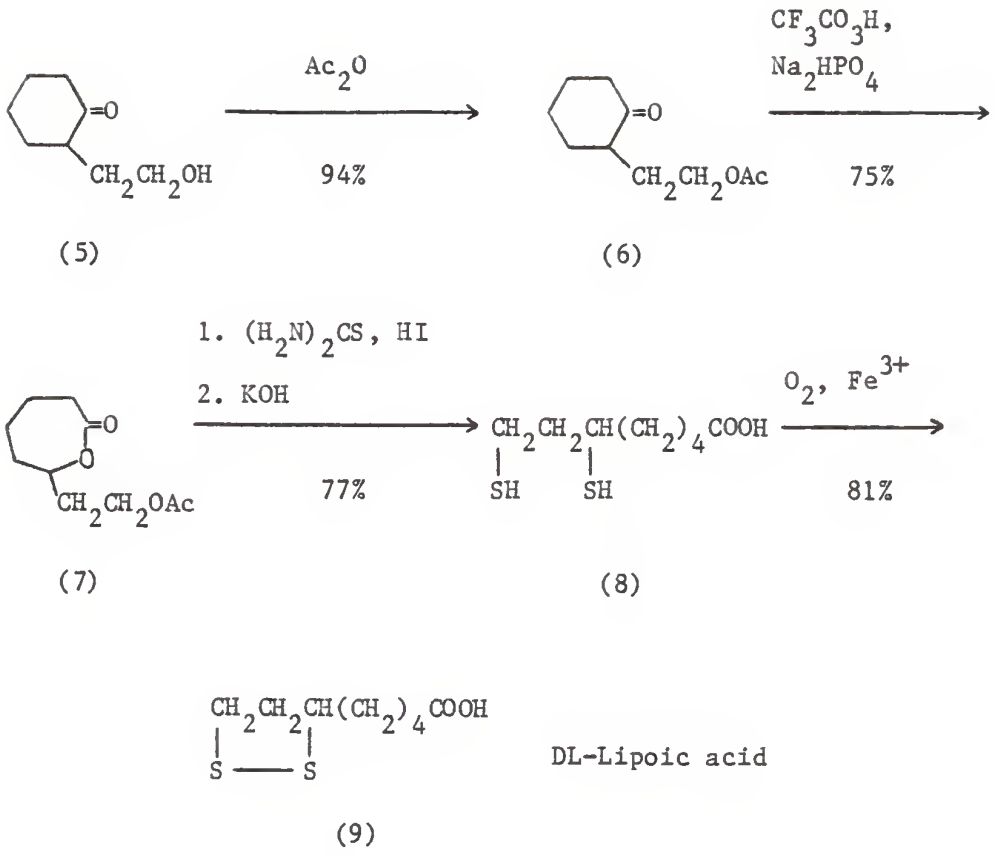


Figure 7. Part II of lipoic acid synthesis.

1—6-lactone (7). The reaction was performed by addition of trifluoroperacetic acid to the cold (-5°) slurried disodium phosphate and ketone in methylene chloride. The crude yield gave negative test with 2,4-dinitrophenylhydrazine, suggesting that the oxidation was completed succesfully without leaving any trace of ketone. Segre et al.[6] prepared the above lactone (7) by oxidizing the ketone (6) with peracetic acid over 24hr to yield 65% Of distilled product; however our attempts to duplicate Segre's method resulted in yields lower than 50%.

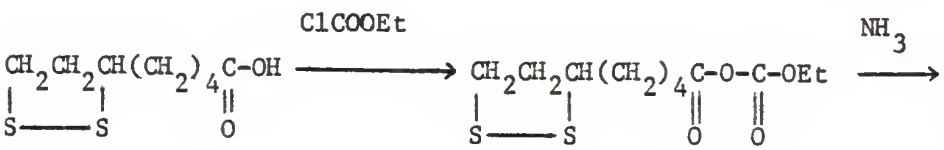
In general, lactones treated with thiourea in strong

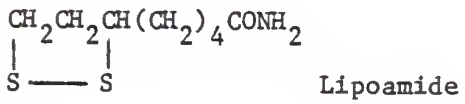
mineral acid, followed by alkaline hydrolysis of the thiouronium salt thus obtained, yield thiol-acid.[10,11] In fact from our lactone (7), we easily obtained dihydrolipoic acid (8) in 77% yield, which, after oxidation, gave 81% of lipoic acid (9), with the same known infrared spectrum [18]. The overall yield of this acid was 27.5% from ethyl bromoacetate. This compares to 20.4% as obtained by Segre[6] from cyclohexanone. While this appears to be an improper comparison because we are not counting the preparation of enamine, we need to point out that Segre used an excess amount of ethyl bromoacetate while in our case, for labeling purposes, a limited amount of ethyl bromoacetate and an excess amount of enamine was used. Our reaction sequence shows an increase of 7.1% in overall yield; this corresponds to a 33.5% increase in the amount of product isolated. The yield in Segre's synthesis was lower, probably due to more numerous isolations (8) and reactions (11). Comparatively, our method of preparation has the advantages of requiring fewer steps with only 10 reactions and 5 isolations, of consuming less time and of obtaining higher yield. The goal of finding a very short synthesis however, was not acheived.

In attempting to synthesize the $^{13}C_{8,7}$-labeled lipoic acid, we chose 1g of ethyl bromoacetate as a convenient but minimum amount of material for our synthesis, we anticipated to get .34g of lipoic acid based on the yield of 27.5%. Due to the limited amount of reactant, we carried out the

sequence without any purification and did not obtain the expected amount of lipoic acid but only 30-50 mg of material which was shown to be predominantly lipoic acid by spectral methods. Futhermore the material was tested for biological activity and was shown to be as reactive as 40-50% of pure lipoic acid.

One of the biologically active derivatives of lipoic acid is lipoamide. This amide is a prefered substrate for dihydrolipoic dehydrogenase and has been synthesized as a yellow crystalline solid by reaction of lipoyl chloride or the mixed anhydride of lipoic acid and isobutyl chloroformate, with ammonia.[52,12,13] The latter reaction gives higher yield (82%). We tried similar reactions, but with lipoic acid and ethyl chloroformate, the yield increased up to 90-94%.





Lipoamide

CONCLUSION

In order to study the role of lipoic acid in the reductive acylation, transacetylation, and oxidation reactions catalyzed by the multienzyme complex, we would like to introduce a synthetic pathway to labeled lipoic acid. Comparing to Segre's synthesis, we have improved the yield and reduced the number of synthetic steps and isolation procedures. With this synthetic route one might be able to perform the following labeling on lipoic acid starting with the commercially available isotopic material;

1. ^{13}C-label at position C_7 or C_8 if using either $Br^{13}CH_2COOEt$ ($340/g, 90% enrichment) or $BrCH_2{}^{13}COOEt$ ($245/g, 90% enrichment) in the alkylation step. The ^{13}C-labels are for ^{13}C-NMR detection and there it is desirable to have as much label as possible.
2. ^{3}H or ^{2}H-label at position C_8 if using either $LiAl^{3}H_4$ ($267/25mC, 50-100mC/mmol) or $LiAl^{2}H_4$ ($15/g, 99% enrichment) in the reduction step. ^{3}H-label is needed as much as possible since the decay energy is relatively low (.01861 MeV) while ^{2}H-label is for ^{1}H or ^{2}H-NMR detection and therefore maximum enrichment is required.
3. ^{35}S-label at position C_6 and/or C_8 if using $(H_2N)_2C^{35}S$ ($291/10mC, 10-50mC/mmol) to react with the lactone (7). This label is radioactive; its decay energy is relatively high (.1674 MeV) and therefore only a small amount of label is needed.

One or more of these synthesis will be carried out in the near future.

EXPERIMENTAL SECTION[26]

Preperation of 1-Pyrrolidinocyclohexene. A solution of 49g (.5 mol) of cyclohexanone and 42.6g (.6 mol) of pyrrolidine in 500 ml of dry benzene was refluxed for 4 hrs under nitrogen. The water generated from the reaction was collected via a Dean Stark trap. The benzene and excess pyrrolidine were removed in vacuo and the oily residue was distilled through a 15 cm Vigreux column to yield 72.3g (95.7%) of the enamine; b.p. 98-101° (5mm); lit. b.p. 110-112° (12 mm)[27]; nmr (CCl_4) p pm 4.15 (m, 1H), 2.9 (m, 4H), 1.4-2.3 (m, 12H); ir (thin film in $CHCl_3$) 1660 cm^{-1} (C=C).

Preparation of Ethyl 2-oxocyclohexylacetate. To a solution of 34g (.22 mol) of 1-pyrrolidinocyclohexene in 500 ml of dry methanol was added dropwise over a period of 40 min 25g (.15 mol) of ethyl bromoacetate. Refluxing and stirring were continued for another 2 hour. Water (30 ml) was added through condenser and heating was continued for another 2 hr. Methanol was removed in vacuo, the residue was treated with 15 ml of water and extracted with 3 x 20 ml of ether. The combined extracts were washed with dilute acid, then with water, dried over magnesium sulfate, and evaporated. The residual oil was distilled through a 15 cm column to yield 20.15g (73%) of the desired product; b.p. 105-107° (3.5 mm); lit. b.p. 95-101° (1.5 mm)[28]; ir (neat) 1710 (C=O, ketone) and 1730 cm^{-1} (C=O, ester); nmr (CCl_4) ppm 4.1 (q, 2H), 1.4-2.9 (m, 11H), 1.2 (t, 3H).

Preparation of Ethyl (2-pyrrolidinocyclohexenyl)acetate. A solution of 7.3g (.04 mol) of ethyl 2-oxocyclohexylacetate and 4.2g (.059 mol) of pyrrolidine in 50 ml of dry benzene was refluxed for 2 days under nitrogen. A few crystal of para-toluenesulfonic acid was added as catalyst. The water generated from the reaction was collected in a Dean Stark trap. The benzene and excess pyrrolidine were removed in vacuo and the residue was distilled to yield 7.9g (83.3%) of the liquid enamine; b.p. 95-100° (.5 mm); nmr (CCl_4) ppm 4.02 (q, 2H), 2.85 (m, 4H), 2.41 (m, 2H), 1.80 (m, 12H), 1.15 (t, 3H); ir 1740 (C=O, ester) and 1650 (C=C, enamine).

Preparation of 2-(2-Hydroxyethyl)cyclohexanone. To a stirred suspension of .6g (.016 mol) of lithium aluminum hydride in 50 ml of ether under nitrogen at room temperature was added dropwise 3.75g (.016 mol) of ethyl (2-pyrrolidinocyclohexenyl) acetate in 10 ml of dry ether over a period of 20 min. The mixture was refluxed for another 20 min. The excess reagent was destroyed with the addition of .6 ml of water, .6 ml of 15% aqueous sodium hydroxide and again 1.8 ml of water. The solution was filtered, solvent removed in vacuo; the resulting oil was treated with 10 ml of aqueous methanol and mixture refluxed for 2 hrs. Methanol was removed in vacuo, residue diluted with 3 ml of water, extracted with ether; the combined extracts were washed with 2N hydrochloric acid, 5% sodium bicarbonate and water, dried over sodium sulfate and evaporated. The residue was distilled to yield 2.0g (88%) of 2-(2-hydroxyethyl)cyclohexanone b.p. 105-110° (3 mm), lit. b.p. 68-70° (.25 mm)[29]; ir (neat)

3400 (broad, O-H) and 1710 cm^{-1} (strong, C=O); nmr (CCl_4) ppm 3.3-4.1 (m, 3H), upon shaking with D_2O, the multiplet at 3.5 dissappeared; the recrystallized 2,4-dinitrophenylhydrazone from aqueous ethanol melted at 145-147°, lit. m.p. 148-149°[29].

Preparation of 2-(2-Hydroxyethyl)cyclohexanone from ethyl bromoacetate. To a stirred solution of 10g (.066 mol) of 1-pyrrolidinocyclohexene in 60 ml of dry benzene under nitrogen was added dropwise a solution of 7.5g (.045 mol) of ethyl bromoacetate in 30 ml of benzene for 15 min. Heating and stirring were continued for 2 hr. The solution was allowed to cool to room temperature, 6.8 g (.045 mol) of 1,5-diazabicyclo [5.4.0] undec-5-ene (DBU) was added and stirring was continued for 30 min. The solution was filtered under a nitrogen atmosphere, residue washed with ether. The resulting filtrate was added dropwise to a stirred suspension of 1.5g (.04 mol) of lithium aluminum hydride in 60 ml of ether at room temperature for 15 min. Stirring was continued for another 15 min. The excess reagent was destroyed with the successive addition of 1.5 ml of water, 1.5 ml of 15% aqueous sodium hydroxide and again 4.5 ml of water. The solvent was filtered and removed in vacuo. The resulting oil was treated with 30 ml of 20% aqueous methanol and mixture refluxed for 2 hr. Methanol was removed in vacuo, residue diluted with 9 ml of water, extracted with ether, washed with dilute acid, 5% sodium bicarbonate and water, dried over sodium sulfate and evaporated . The residue was distilled to yield 3.99g (62.5%) of the desired 2-(2-Hydroxyethyl)cyclohexanone.

Preparation of 2-(2-Oxocyclohexyl)ethyl acetate. A mixture of 3g (.021 mol) of 2-(2-hydroxyethyl)cyclohexanone and 4.3g (.04 mol) of acetic anhydride in 10 ml of pyridine was stirred at 50-60° for 1 hr. The solution was poured in ice water and extracted with ether. The combined extracts were washed with 5% hydrochloric acid, 5% sodium bicarbonate and water, dried over sodium sulfate, and evaporated. Distillation under reduced pressure gave 3.66g (94%); b.p. 105-110° (3 mm), lit. b.p. 142-146° (15 mm)[6]; ir (neat) 1740 (C=O, ester), 1715 (C=O, ketone) and 1235 cm^{-1} (C-O); nmr (CCl_4) ppm 4.05 (t, 2H), 2.05 (s, 3H). The 2,4-dinitrophenylhydrazone formed yellow needles from ethanol, m.p. 110-111°, lit. m.p. 109-111°, 111-112°[6, 29].

Attempted preparation of 2-(2-Oxocyclohexyl)ethyl trifluoroacetate. To a solution of 1g (.007 mol) of 2-(2-hydroxyethyl)cyclohexanone in 10 ml of methylene chloride at -5° was added slowly a solution of 2.1g (.01 mol) of trifluoroacetic anhydride in 5 ml of methylene chloride. Stirring was continued for 30 min. Solvent, excess reagent and by-product were removed in vacuo, keeping the bath temperature below 25°. A yellow oil was left behind and the nmr of this material indicated a mixture of several products.

Preparation of 2-(2-Oxocyclohexyl)ethyl 3,5-dinitrobenzoate. A mixture of .5g (.0035 mol) of 2-(2-hydroxyethyl)cyclohexanone and .8g (.0035 mol) of 3,5-dinitrobenzoyl chloride in 2 ml of pyridine and 4 ml of benzene was refluxed for 30 min, treated with 5 ml of water and extracted several times with benzene. The combined extracts were washed with 5% hydrochloric acid, 5% sodium

bicarbonate and water, dried over magnesium sulfate and evaporated A yellow oil (1.05g) was left, which did not seem to recrystallize in aqueous methanol, ethanol, n-propanol, carbon tetrachloride and heptane-benzene mixture; ir (neat) 3040 (C=C-H), 1730 (C=O,ester), 1715 (C=O, ketone), 1535 and 1310 cm^{-1} (strong, NO_2); nmr (CCl_4) ppm 7.55 (s, 3H), 4.45 (t, 2H).

Preparation of 6-Hydroxy-8-acetoxyoctanoic acid 1—6-lactone. A mixture of .65 ml (.027 mol) of 98% hydrogen peroxide and 6.9g (.033 mol) of trifluoroacetic anhydride in 20 ml of chloroform was stirred at room temperature for 2 hr and was then added to a stirred suspension of 3.3g (.018 mol) of 2-(2-oxocyclohexyl)-ethyl acetate and 11g of disodium phosphate in 20 ml of methylene chloride at -5° for 20 min. The mixture was allowed to warm to room temperature, stirred for 1 hr, filtered, washed with 10% sodium bicarbonate and water, dried over magnesium sulfate and evaporated. The pale yellow oil was distilled to yield 2.69g (75%) of the desired lactone; b.p. 122-128° (.4 mm), lit.b.p. 125-130° (.5 mm)[6]; ir (neat) 1740 and 1730 cm^{-1} (2 C=O); nmr (CCl_4) ppm 4.15 (m, 3H), 2.1 (s, 3H), no reaction with 2,4-dinitrophenylhydrazine.

Attempted oxidation of 2-(2-Hydroxyethyl)cyclohexanone. Same procedure as above; several attempts have been done, the reactions were carried out at -10° and 0°, peracetic acid was also tried, the resulting products could not be identified.

Attempted esterification and oxidation of 2-(2-Hydroxyethyl)cyclohexanone by trifluoroacetic anhydride and trifluoroperacetic acid. To a solution of 1g (.007 mol) of 2-(2-hydroxyethyl)cyclohexanone in 2 ml of chloroform at 0° was added dropwise a solution of 1.7g (.0084 mol) of trifluoroacetic anhydride in 4 ml of chloroform for 20 min. The mixture was stirred for another 10 min and 9 ml of trifluoroperacetic acid in chloroform was added dropwise for 10 min at 0°; stirring was continued for 20 min. Solvent was removed in vacuo, keeping the bath temperature below 25°. A yellow oil was left behind, which could not be identified. Several changes have been done in vain such as dropping the temperature to -10° and using methylene chloride as solvent.

Preparation of Dihydrolipoic acid. A mixture of 2.5g (.0215 mol) of 6-hydroxy-8-acetoxyoctanoic acid 1—6-lactone, 7.4g (.097 mol) of thiourea and 9.84g (.044 mol) of 57% hydroiodic acid was refluxed for 36 hr and then hydrolysed with 40 ml of 30% aqueous potassium hydroxide in the dark under nitrogen for 12 hr. The reaction mixture was extracted with ether, the aqueous layer separated, acidified with 2N hydrochloric acid and extracted with chloroform. The combined extracts were washed with water, dried over magnesium sulfate and evaporated. The residual oil was distilled to yield 2.0g (77%) of dihydrolipoic acid; b.p. 150-155° (.2 mm), lit. b.p. (bath temperature) 180° (1.5 mm)[6]; ir (neat) 3300 (broad, COOH), 1710 (C=O), 2570 (S-H), 1285 and 1235 cm^{-1} (carboxylic O-H); nmr (CCl_4) ppm 10.2 (s, 1H).

Preparation of lipoic acid. Dihydrolipoic acid (1g, .0048 mol) was dissolved in 1 ml of 15% sodium hydroxide, adjusted with 2N hydrochloric acid to pH 8-9, diluted with 15 ml of water containing .3 ml of .2N ferric chloride hexahydrate, through which a slow stream of oxygen was bubled for 24 hr. The resulting solution was washed with chloroform, acidified with 2N hydrochloric acid, extracted with warm chloroform and this was washed with water, dried over sodium sulfate and evaporated. The yellow oil was recrystallized from heptane to yield .8g (81%) of lipoic acid; m.p. 60-61°, lit. m.p. 60[23], 61[15], 61-62[6,18]; ir (KBr) 3300 (broad, COOH) and 1700 cm^{-1} (C=O); nmr (CCl_4) ppm 11.1 (s, 1H), 3.5 (m, 1H), 3.15 (m, 2H), 2.45 (m, 6H) and 1.6 (m, 4H).

Preparation of lipoamide. To a stirred solution of .5g (.0024 mol) of lipoic acid and .247g (.0024 mol) of triethylamine in 7 ml of dry tetrahydrofuran (THF) at -5° was added dropwise a solution of .3g (.0028 mol) of ethyl chloroformate in 2 ml of THF. Stirring was continued for 10 min longer; 2 ml of THF saturated with ammonia was added and a stream of ammonia was passed through the reaction mixture for 10 min at -5°. The mixture was allowed to stand to 25°, solvent removed in vacuo, extracted with chloroform and this was washed with 5% hydrochloric acid, water, 5% sodium bicarbonate, water and brine solution, dried over sodium sulfate and evaporated. The crude yield .47g (94%), m.p. 128-130 , was recrytallized from chloroform-heptane mixture to yield .45g (90%), m.p. 130-131°, lit. m.p. 129.5-132°[12]; ir (KBr) 3320, 3100 (N-H), 1640 (C=O, amide); nmr ($CDCl_3$) ppm 5.7

(broad, 2H), 3.6 (m, 1H), 3.15 (m, 2H), 2.25-1.60 (m, 10H).

Preparation of Octanamide. Same procedure as above; starting with octanoic acid, the yield of octanamide was 90%, m.p. 104-105°, lit. m.p. 105.9° (freezing), 110°[30]; ir (KBr) 3370, 3150 (N-H) and 1640 cm^{-1} (C=O); nmr ($CDCl_3$) ppm 4.7 (2H), 2.25 (2H).

Preparation of N-Methyl octanamide. To a solution of 3.0g (.021 mol) of octanoic acid and 2.02g (.02 mol) of triethylamine in 7 ml of dry tetrahydrofuran (THF) at -5 was added dropwise a solution of 2.7g (.025 mol) of ethyl chloroformate in 15 ml of THF. Stirring was continued for 10 min longer, 6g of 40% aqueous methylamine in 2 ml of THF was added dropwise for 5 min. The mixture was stirred for another 10 min, allowed to stand to room temperature, solvent removed in vacuo, extracted with ether and this was washed with 5% hydrochloric acid, 5% sodium bicarbonate, water and brine solution, dried over sodium sulfate and evaporated. An oil was left behind, 2.94g (90%); nmr (CCl_4) ppm 7.56 (s, 1H), 2.75 (d, J= 4.5Hz, 3H), 2.17 (m, 2H); ir (neat) 3350 (N-H) and 1650 cm^{-1} (C=O, amide).

Attempted one-step preparation of 2-(2-Hydroxyethyl)cyclohexanone. To a stirred solution of 7.85g (.05 mol) of 1-pyrrolidinocyclohexene in 25 ml of benzene was added dropwise over a period of 10 min a solution of 6.25g (.05 mol) of 2-bromoethanol in 5 ml of benzene. Heating and stirring were continued under nitrogen

for 19 hr. Water (10 ml) was added through condenser and heating was continued for another 2 hr. Benzene was removed in vacuo, an oil left behind,which did not seem to dissolve in ether at any extent; it was, however, very soluble in water. The product was probably the polymeric polyethylene glycol.

Attempted one-step preparation of 2-(2-Oxocyclohexyl)ethyl acetate. To a stirred solution of 7.55g (.05 mol) of 1-pyrrolidinocyclohexene in 25 ml of benzene was added dropwise over a period of 15 min a solution of 8.35g (.05 mol) of 2-bromoethyl acetate in 15 ml of benzene. Heating was continued for 3 hr. Water (10 ml) was added and heating was continued for 45 min. Benzene was removed in vacuo, residue treated with 5 ml of water, extracted with ether and this was washed with 5% hydrochloric acid, 5% sodium bicarbonate and water, dried over magnesium sulfate and evaporated. An oil was left behind, which could not be identified.

Total synthesis of lipoic acid starting with 1g ethyl bromoacetate. Same procedure as given in previous experimental descrptions, however no purification was done due to the limited amount of material. The crude 2-(2-hydroxyethyl)cyclohexanone was prepared (as in p.24) and acetylated (p.25), followed by an oxidation (p.26) to yield ca. .3-.4g of crude material (supposedly 6-Hydroxy-8-acetoxyoctanoic acid 1—6-lactone). Dihydrolipoic acid was then prepared (p.27) and oxidized (p.28) to yield 30-50 mg of crude lipoic acid which has shown to have a biological activity of 40-50%.[54]

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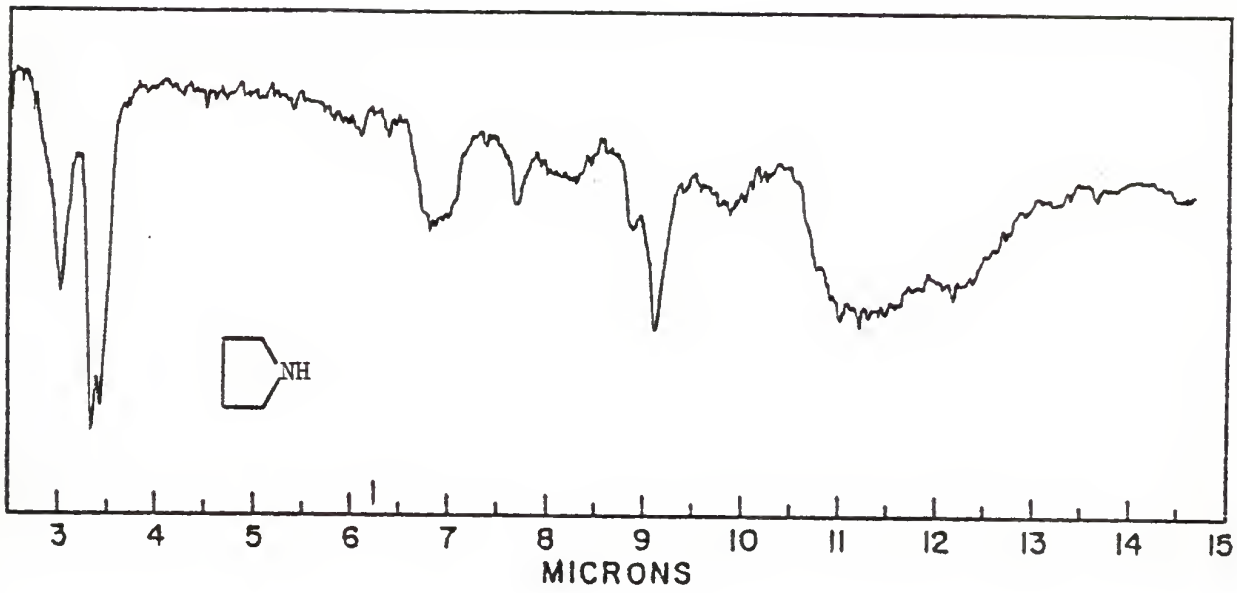
INFRARED SPECTRA

Pyrrolidine

(Thin film)

Cyclohexanone

(Thin film)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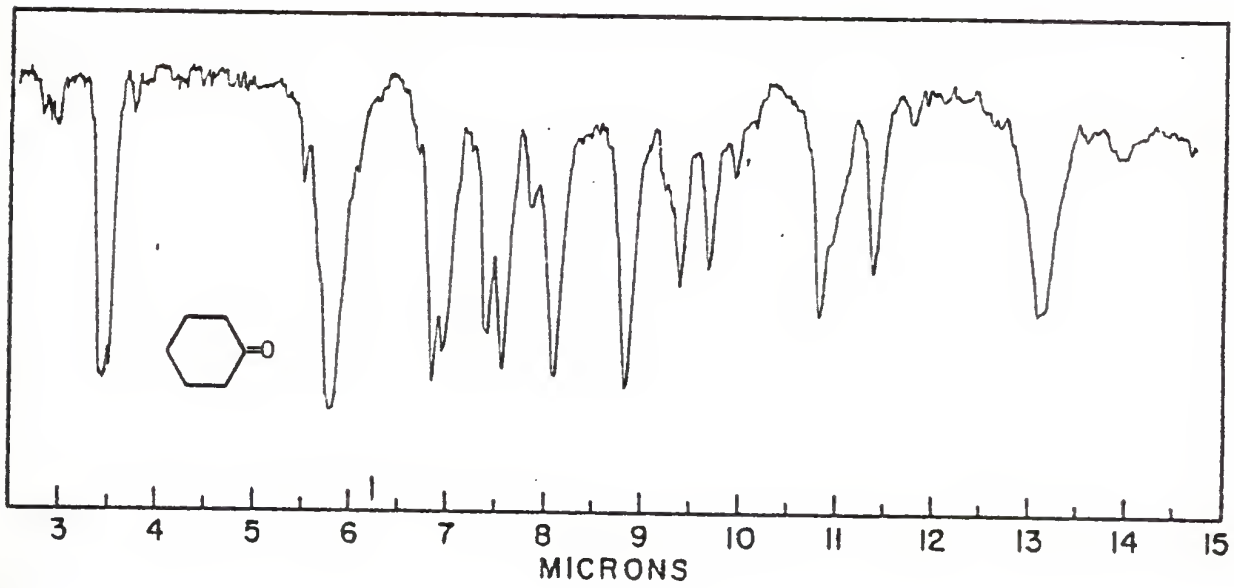
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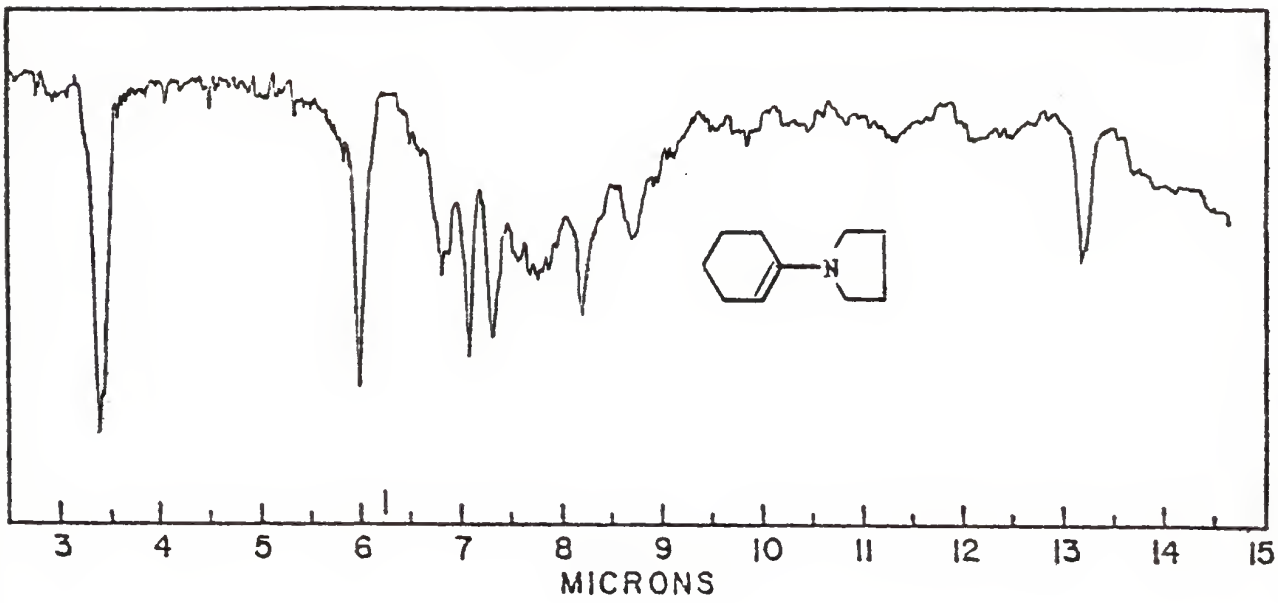
INFRARED SPECTRA

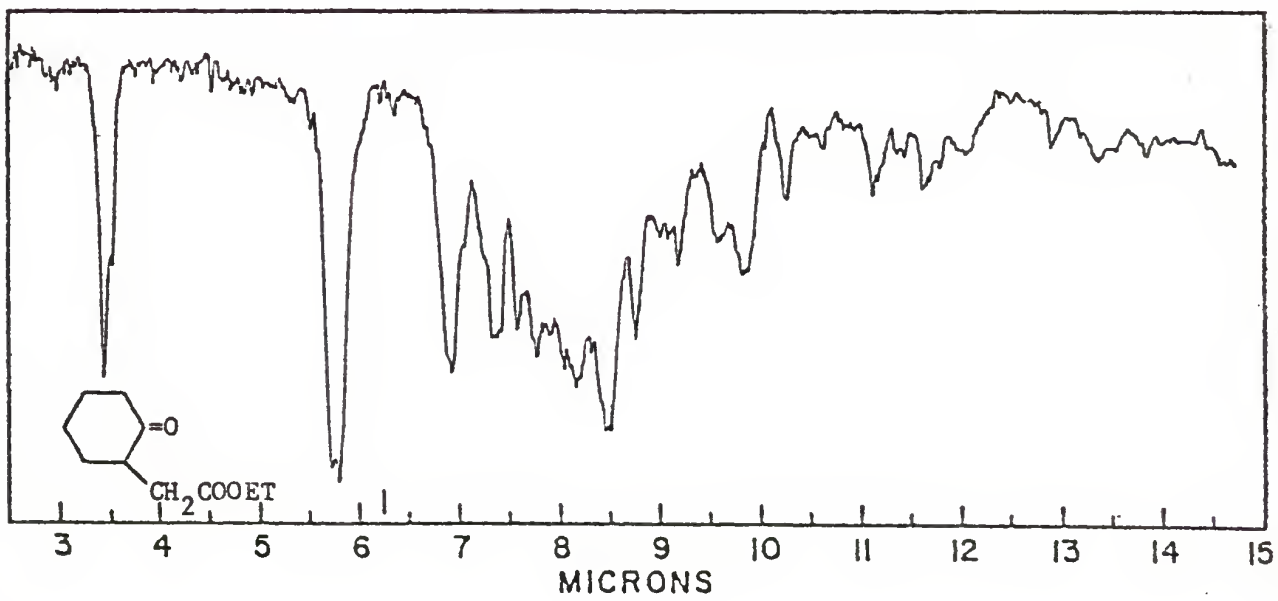
1-Pyrrolidinocyclohexene

(Thin film in $CHCl_3$)

Ethyl 2-oxocyclohexylacetate

(Thin film)


N
3 4 5 6 7 8 9 10 11 12 13 14 15
MICRONS


=O
CH2COOET
3 4 5 6 7 8 9 10 11 12 13 14 15
MICRONS

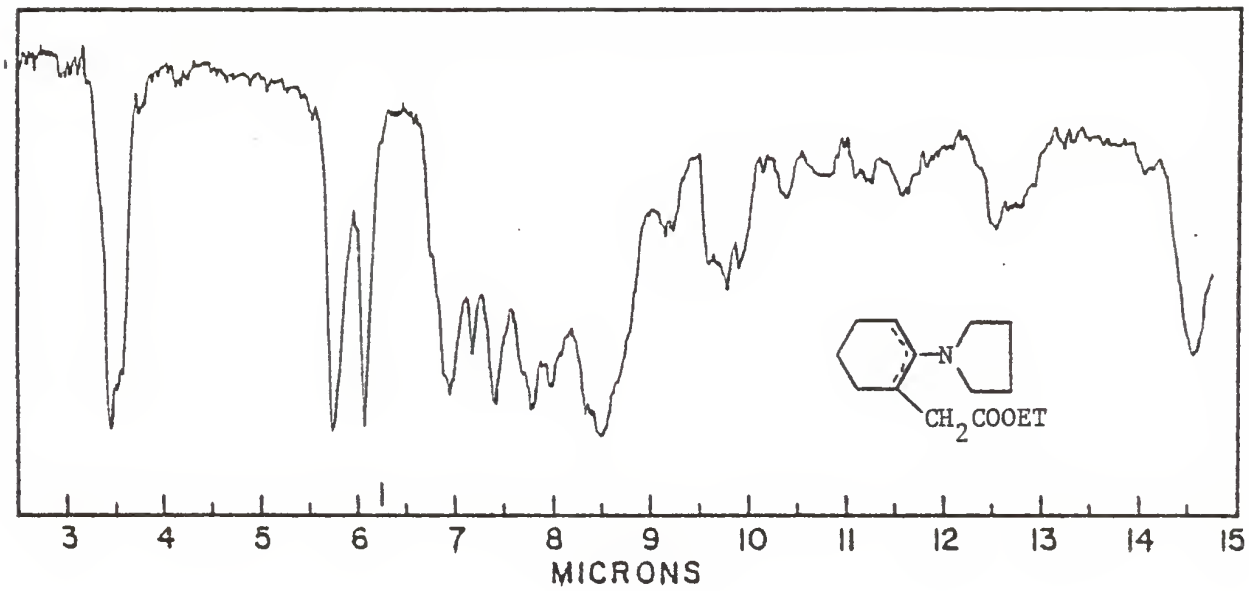
INFRARED SPECTRA

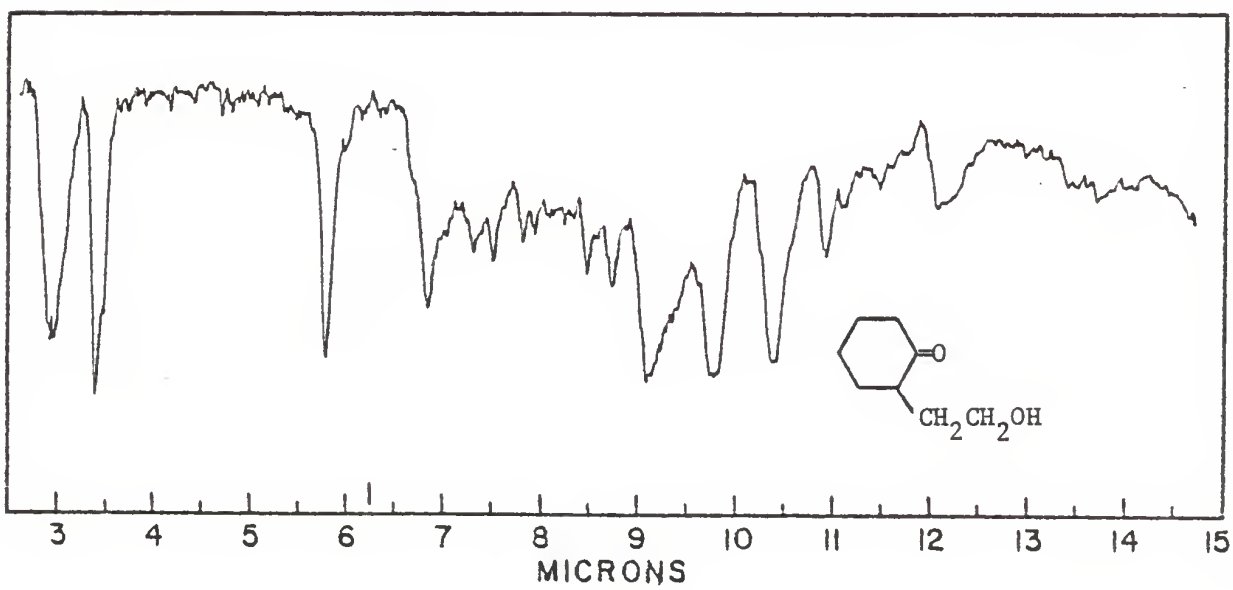
Ethyl (2-pyrrolidinocyclohexenyl)acetate

(Thin film in $CHCl_3$)

2-(2-Hydroxyethyl)cyclohexanone

(Thin film)


N
CH2COOET
3
4
5
6
7
8
9
10
11
12
13
14
15
MICRONS


O
CH2CH2OH
3
4
5
6
7
8
9
10
11
12
13
14
15
MICRONS

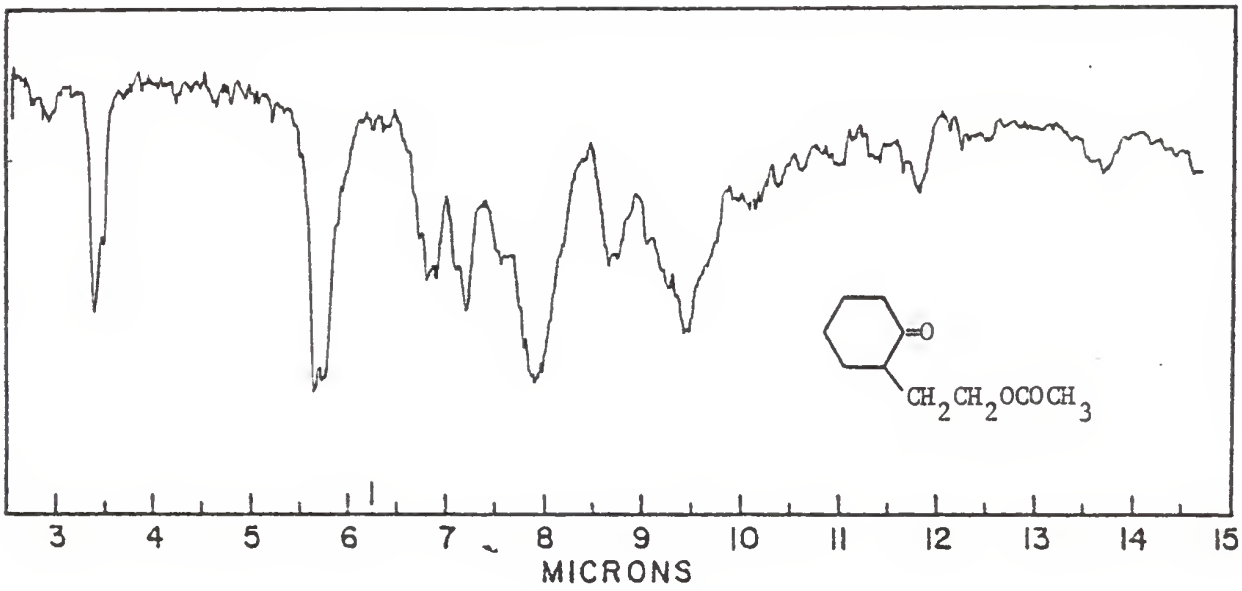
INFRARED SPECTRA

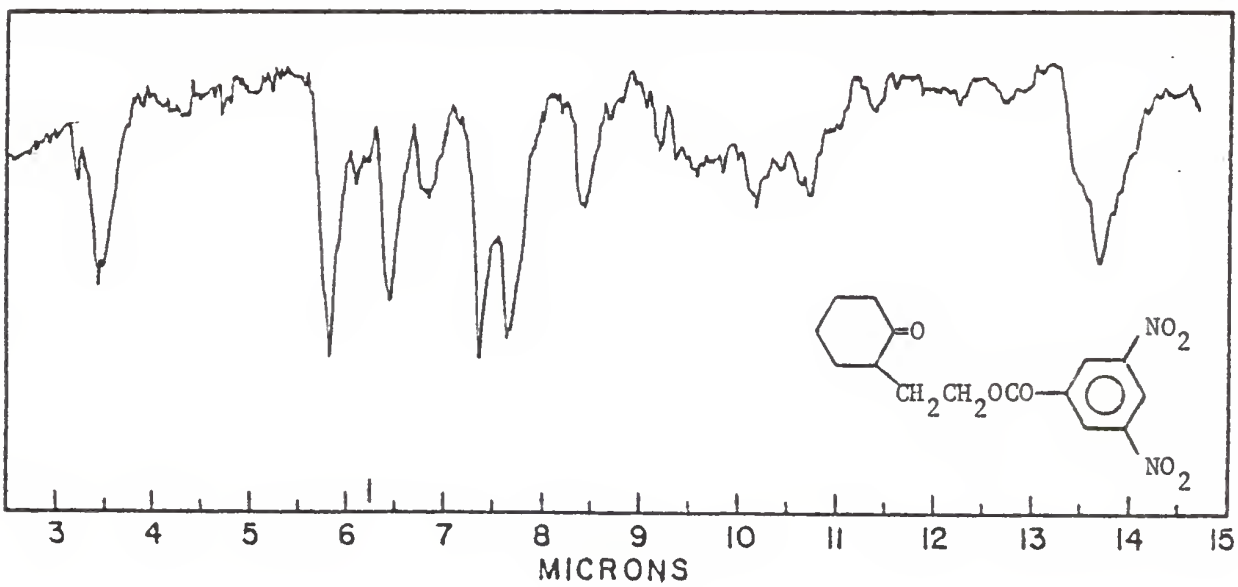
2-(2-Oxocyclohexyl)ethyl acetate

(Thin film)

2-(2-Oxocyclohexyl)ethyl 3,5-dinitrobenzoate

(Thin film)


=O
CH2CH2OCOCH3
3
4
5
6
7
8
9
10
11
12
13
14
15
MICRONS


=O
CH2CH2OCO
NO2
NO2
3
4
5
6
7
8
9
10
11
12
13
14
15
MICRONS

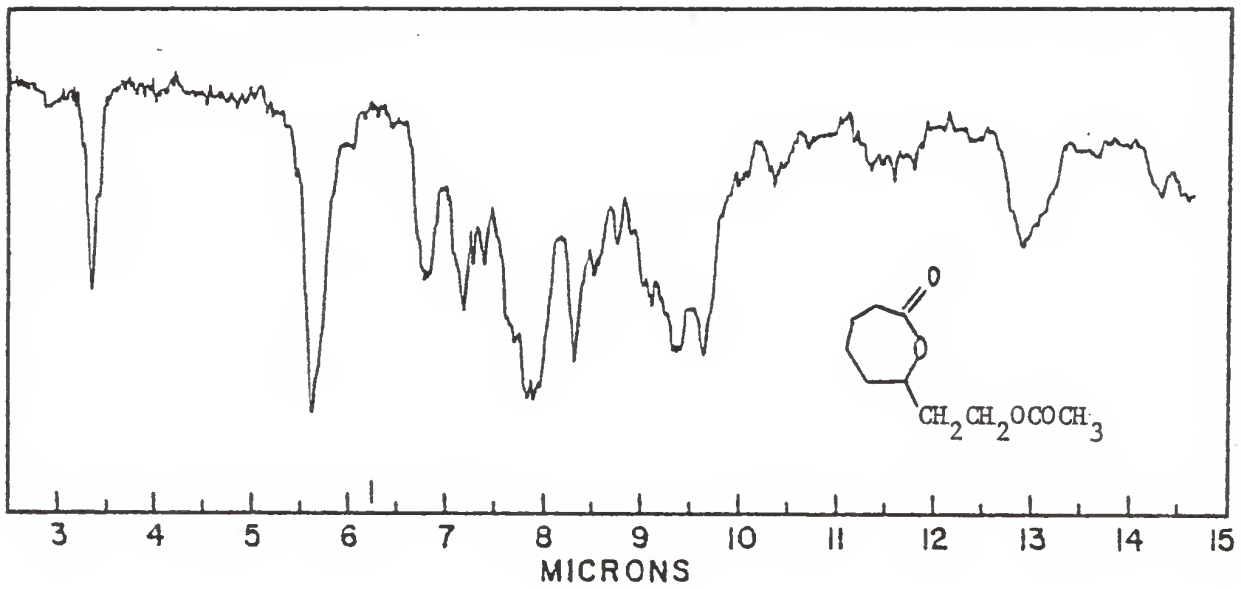
INFRARED SPECTRA

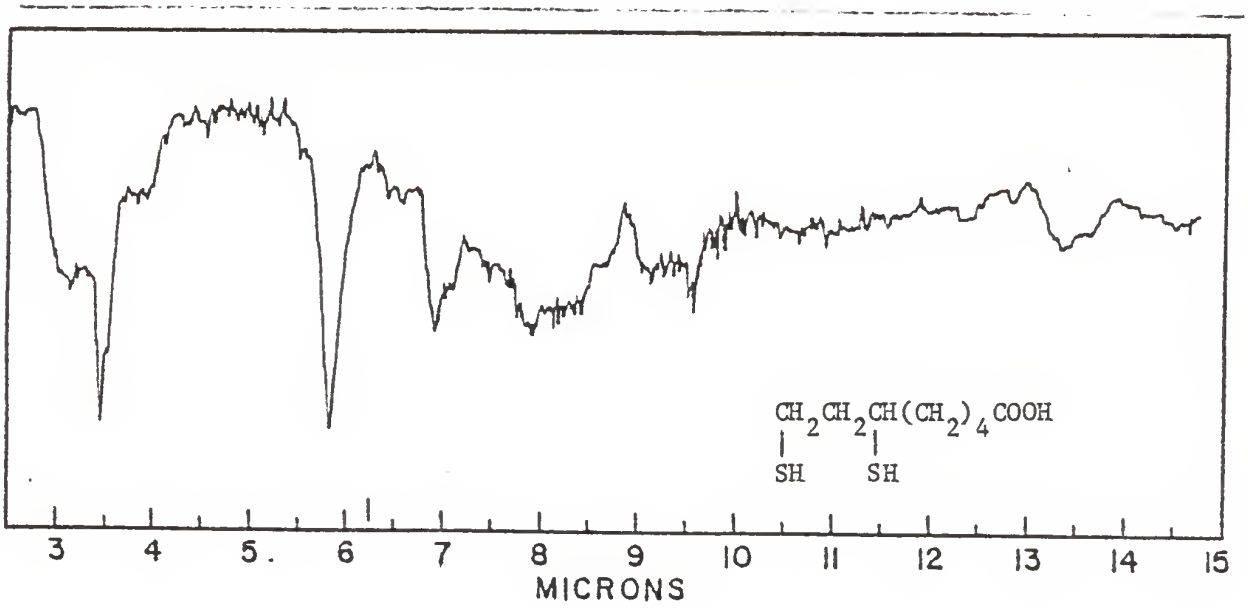
6-Hydroxy-8-acetoxyoctanoic acid 1—6-lactone

(Thin film)

Dihydrolipoic acid

(Thin film)


O
O
CH2CH2OCOCH3
3 4 5 6 7 8 9 10 11 12 13 14 15
MICRONS


CH2CH2CH(CH2)4COOH
SH SH
3 4 5 6 7 8 9 10 11 12 13 14 15
MICRONS

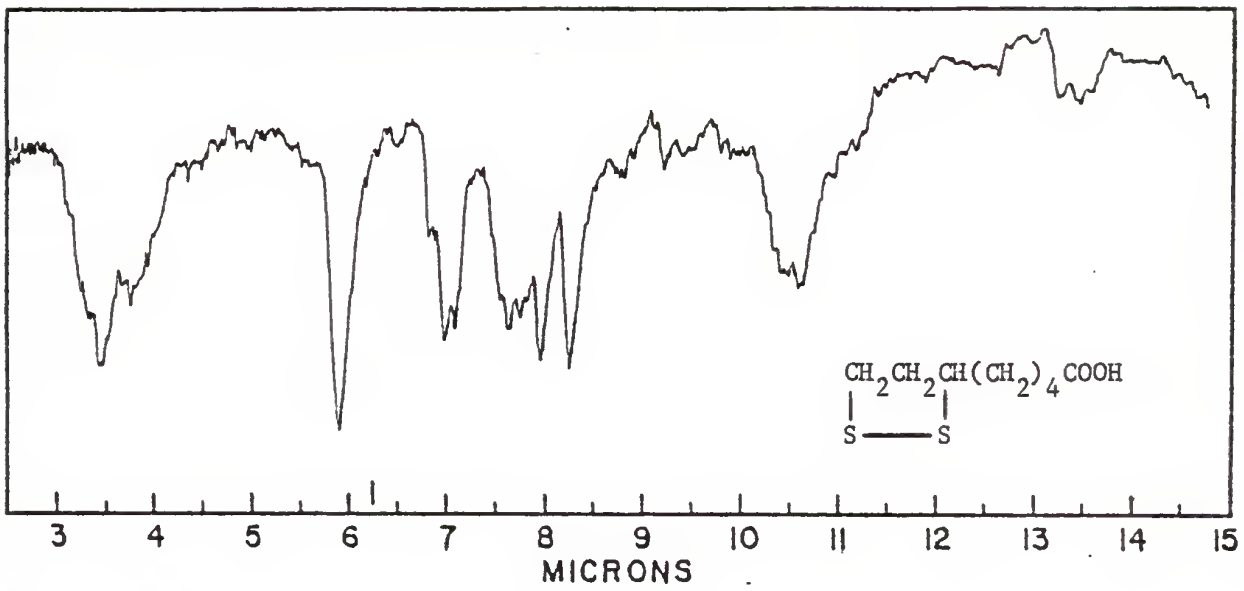
INFRARED SPECTRA

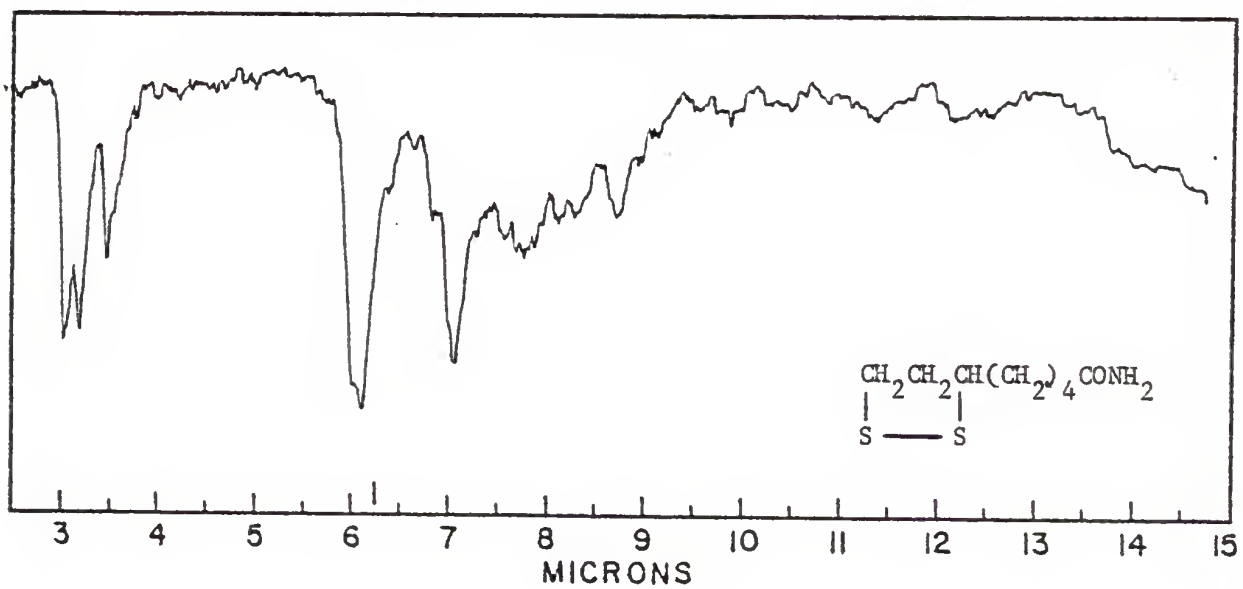
Lipoic acid

(KBr)

Lipoamide

(KBr)


CH2CH2CH(CH2)4COOH
S — S
3 4 5 6 7 8 9 10 11 12 13 14 15
MICRONS


CH2CH2CH(CH2)4CONH2
S — S
3 4 5 6 7 8 9 10 11 12 13 14 15
MICRONS

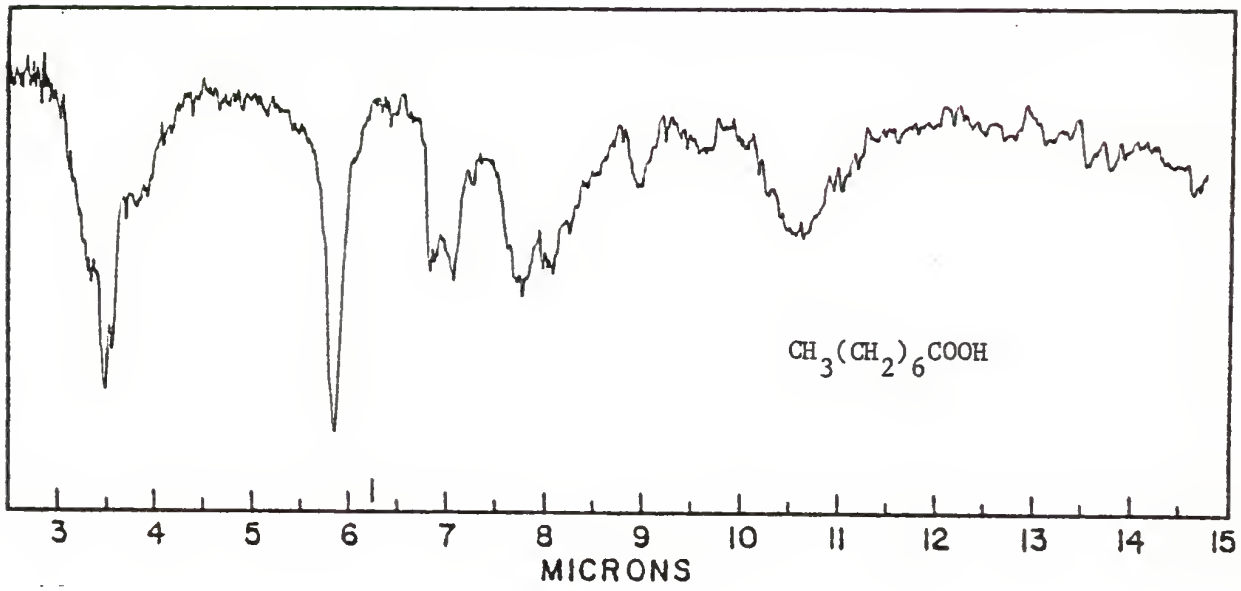
INFRARED SPECTRA

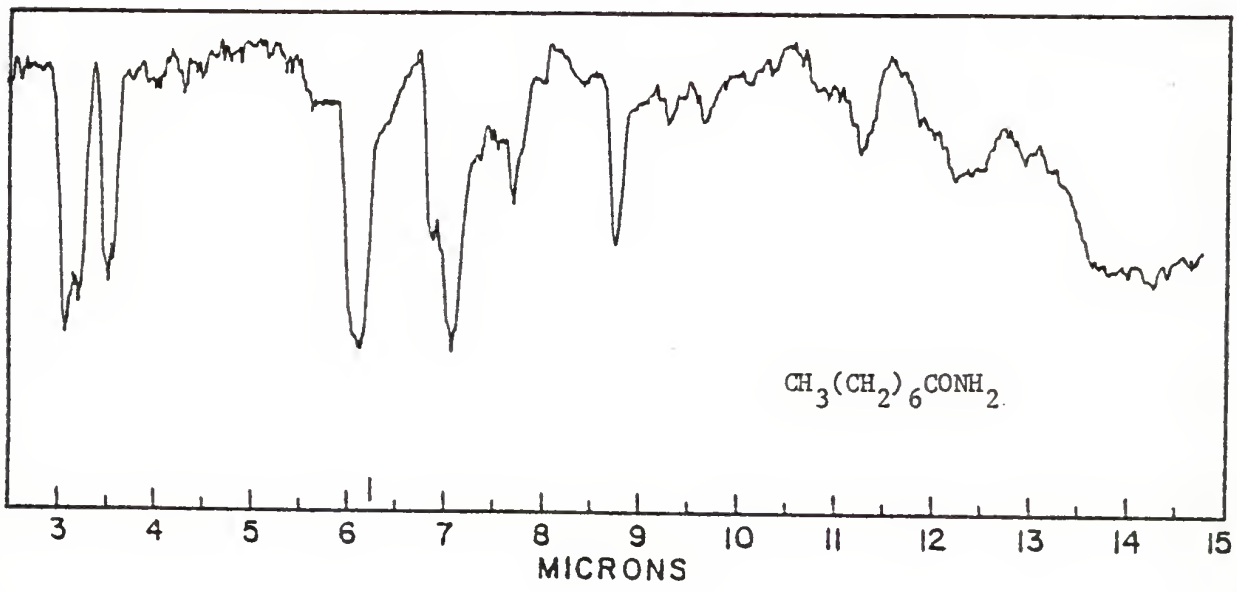
Octanoic acid

(Thin film)

Octanamide

(KBr)

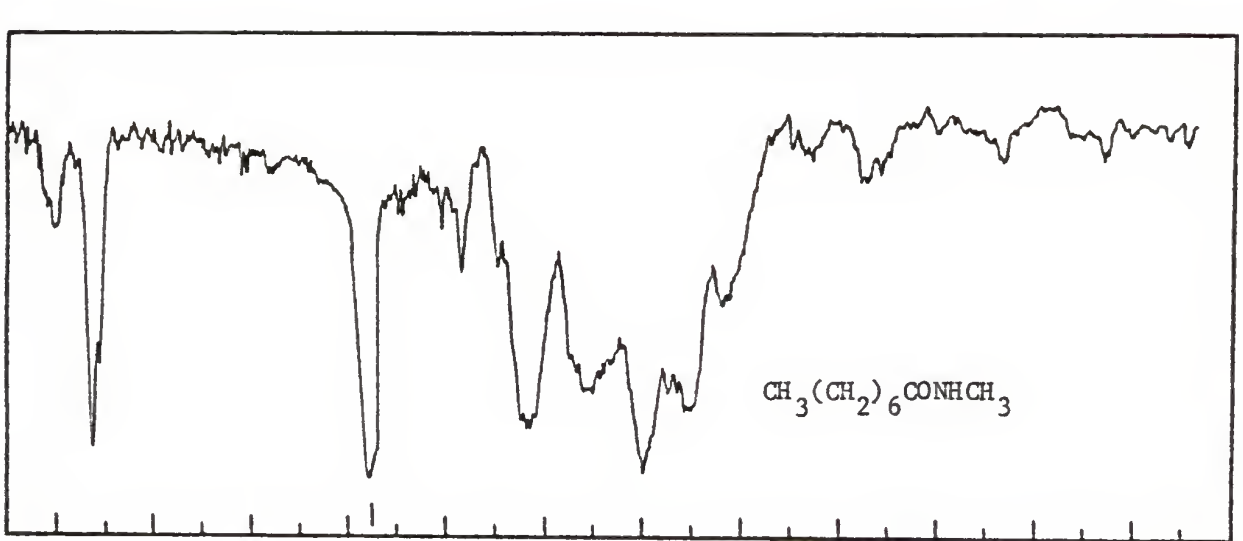

$CH_3(CH_2)_6COOH$
3 4 5 6 7 8 9 10 11 12 13 14 15
MICRONS


$CH_3(CH_2)_6CONH_2$
3 4 5 6 7 8 9 10 11 12 13 14 15
MICRONS

INFRARED SPECTRA

N-Methyl octanamide

(Thin film)


$CH_3(CH_2)_6CONHCH_3$
3
4
5
6
7
8
9
10
11
12
13
14
15
MICRONS

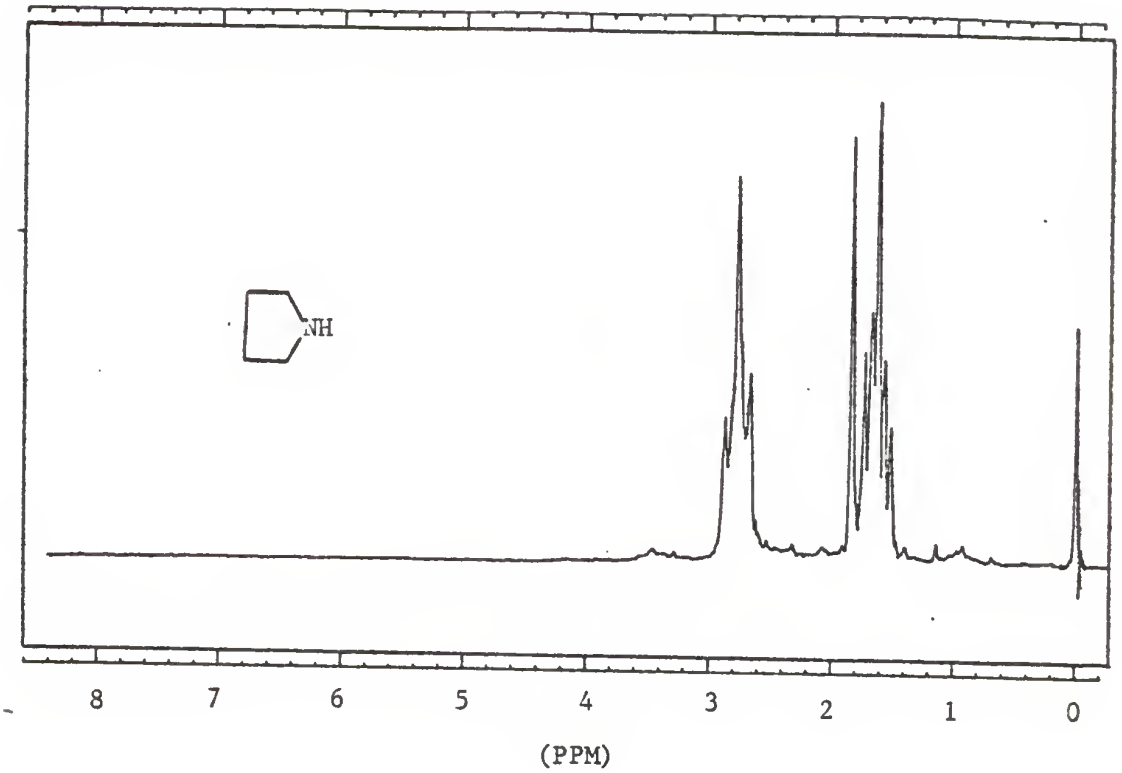
NMR SPECTRA

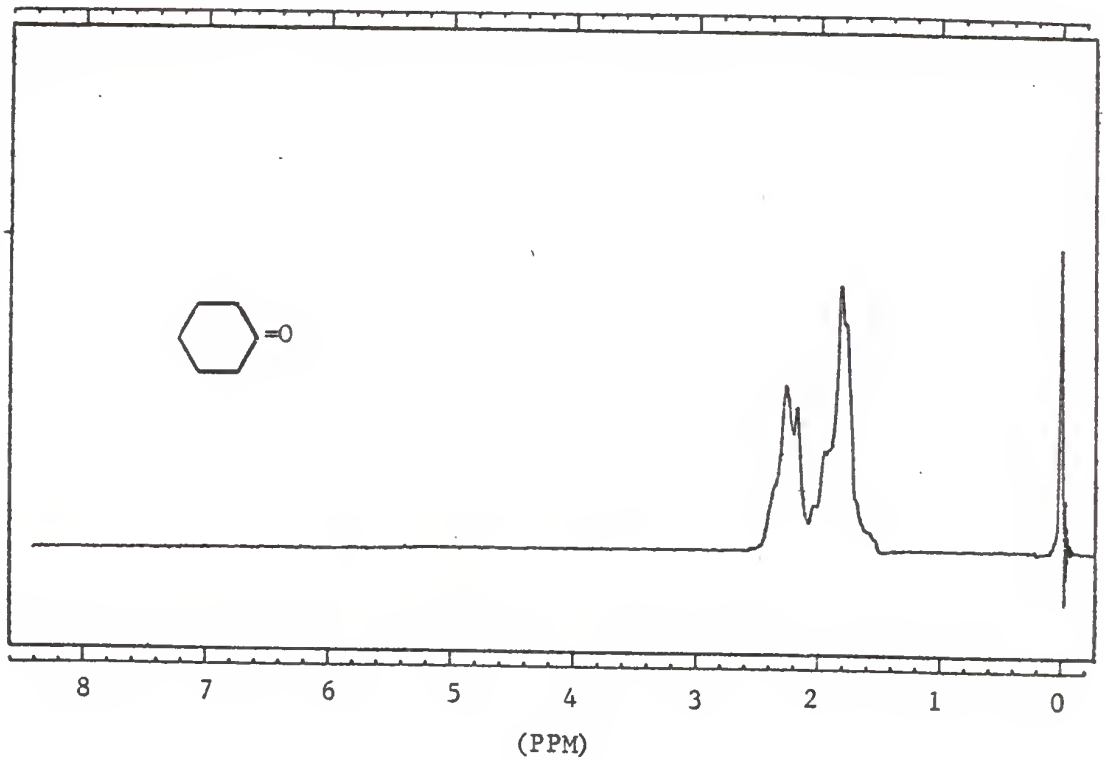
Pyrrolidine

(CCl_4, Internal TMS)

Cyclohexanone

(CCl_4, Internal TMS)


NH
8
7
6
5
4
3
2
1
0
(PPM)


=O
8
7
6
5
4
3
2
1
0
(PPM)

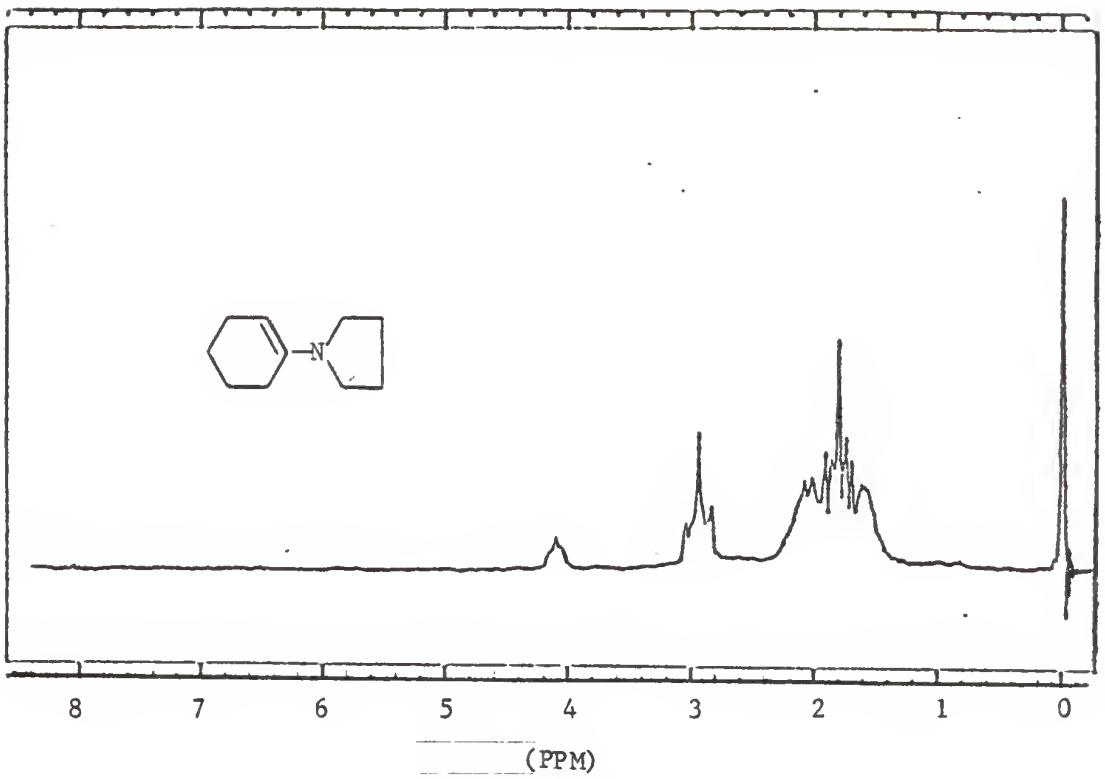
NMR SPECTRA

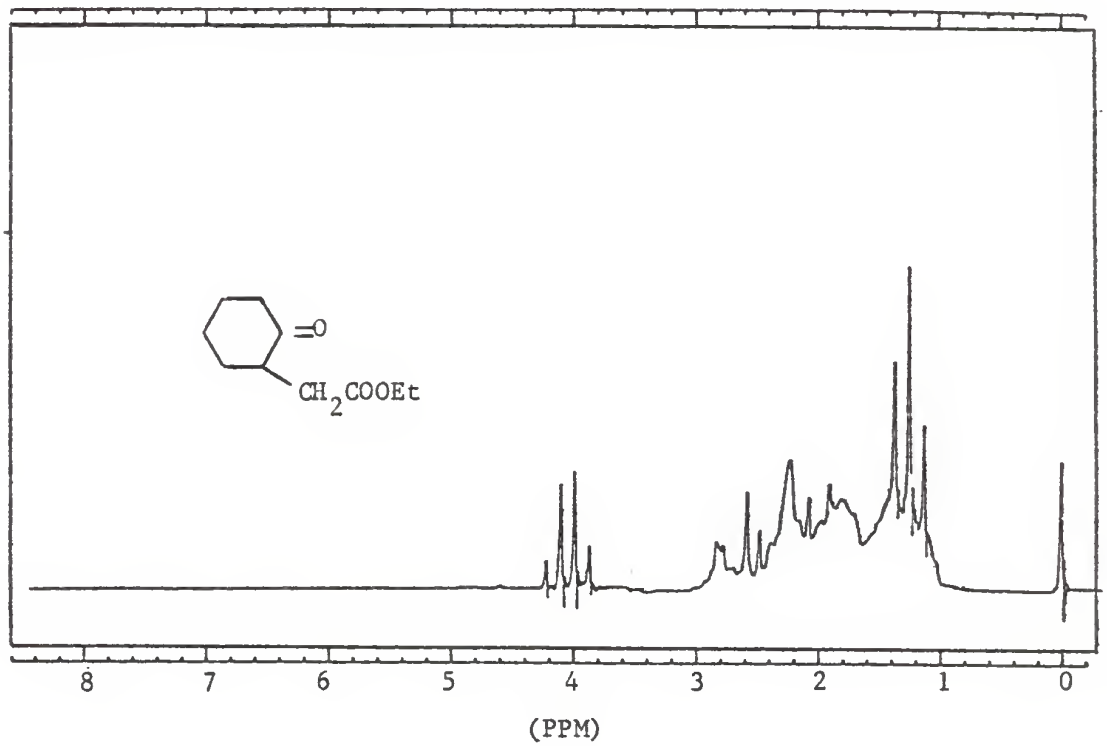
1-Pyrrolidinocyclohexene

(CCl_4, Internal TMS)

Ethyl 2-oxocyclohexylacetate

(CCl_4, Internal TMS)


8
7
6
5
4
3
2
1
0
(PPM)


=O
CH2COOEt
8
7
6
5
4
3
2
1
0
(PPM)

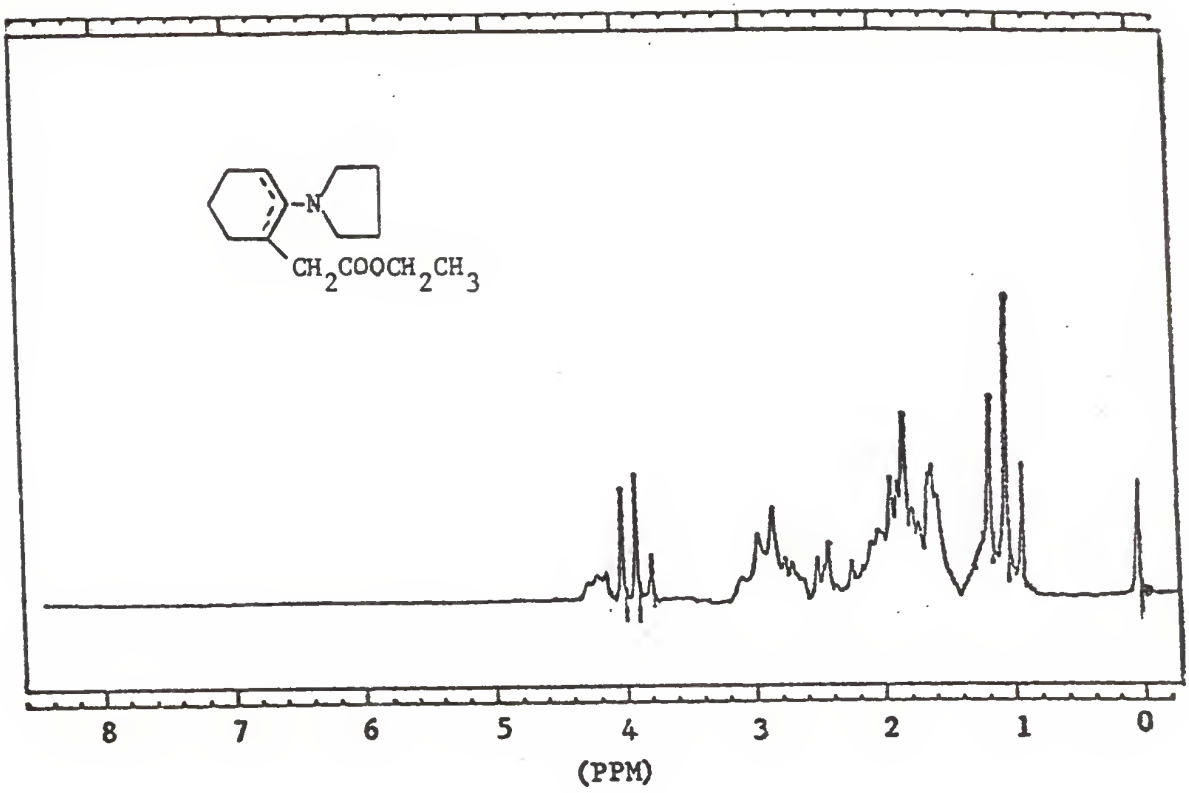
NMR SPECTRA

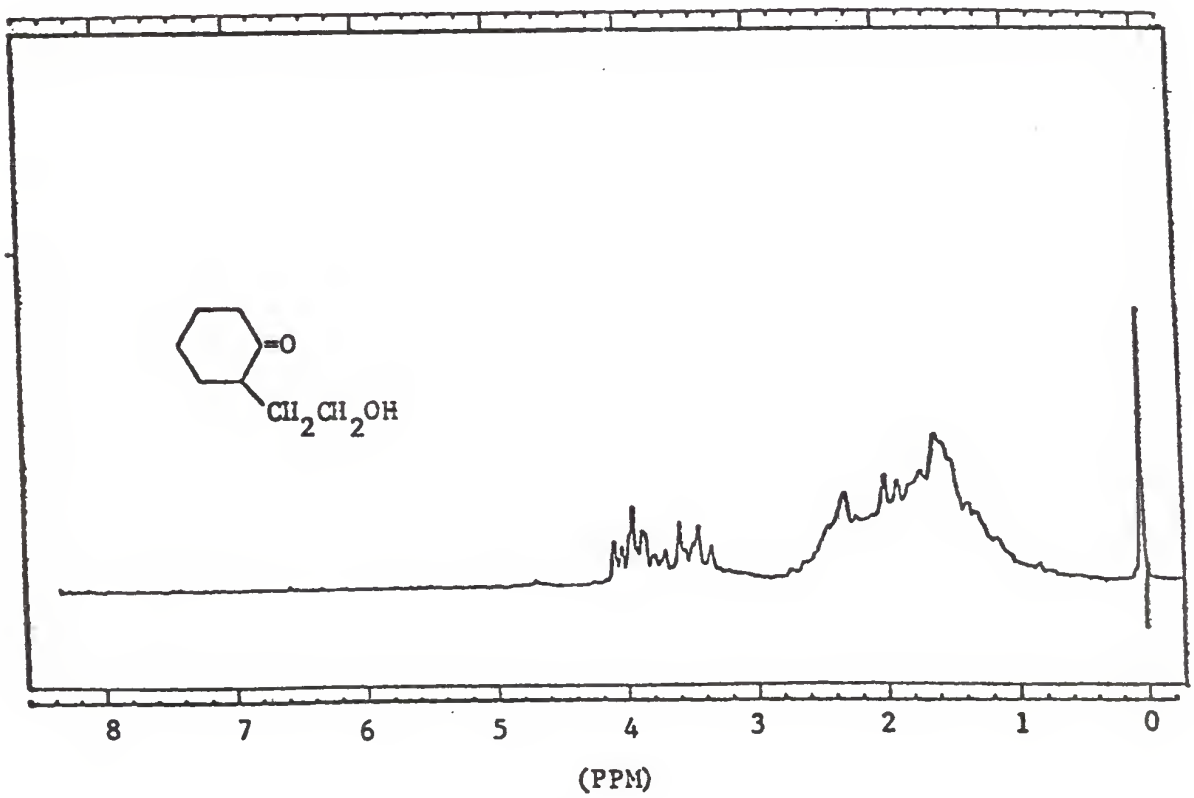
Ethyl (2-pyrrolidinocyclohexenyl)acetate

(CCl_4, Internal TMS)

2-(2-Hydroxyethyl)cyclohexanone

(CCl_4, Internal TMS)


-N
CH2COOCH2CH3
8
7
6
5
4
3
2
1
0
(PPM)


=O
CH2CH2OH
8
7
6
5
4
3
2
1
0
(PPM)

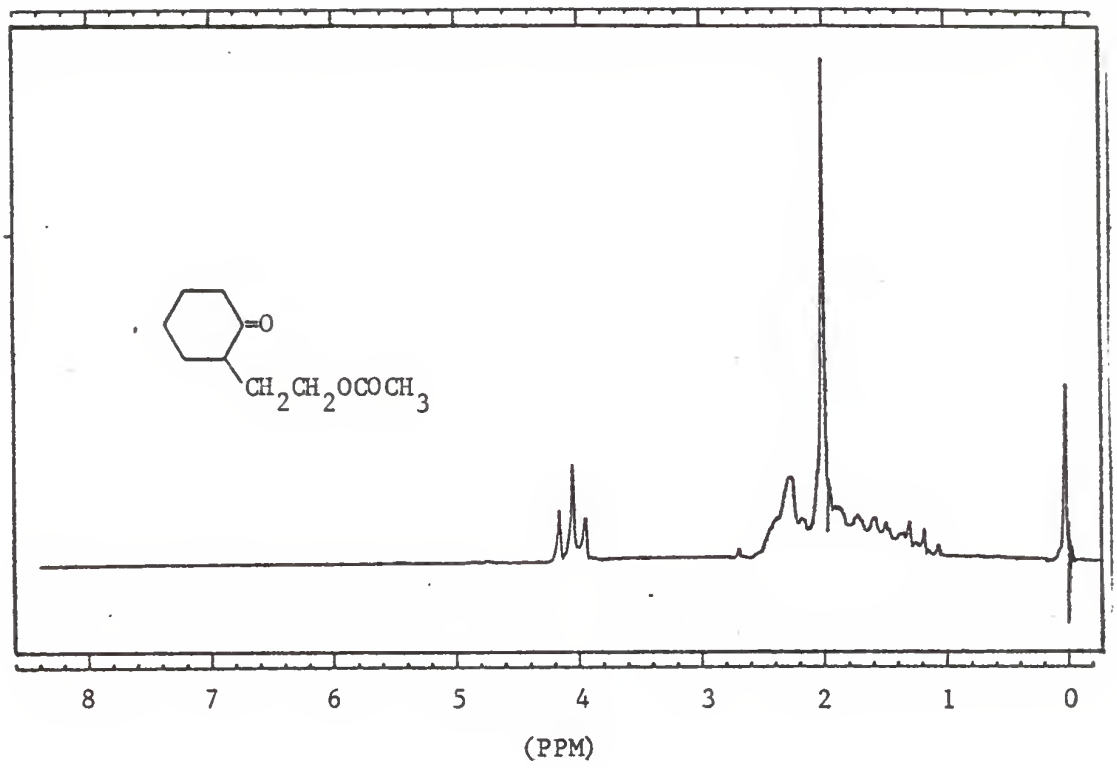
NMR SPECTRA

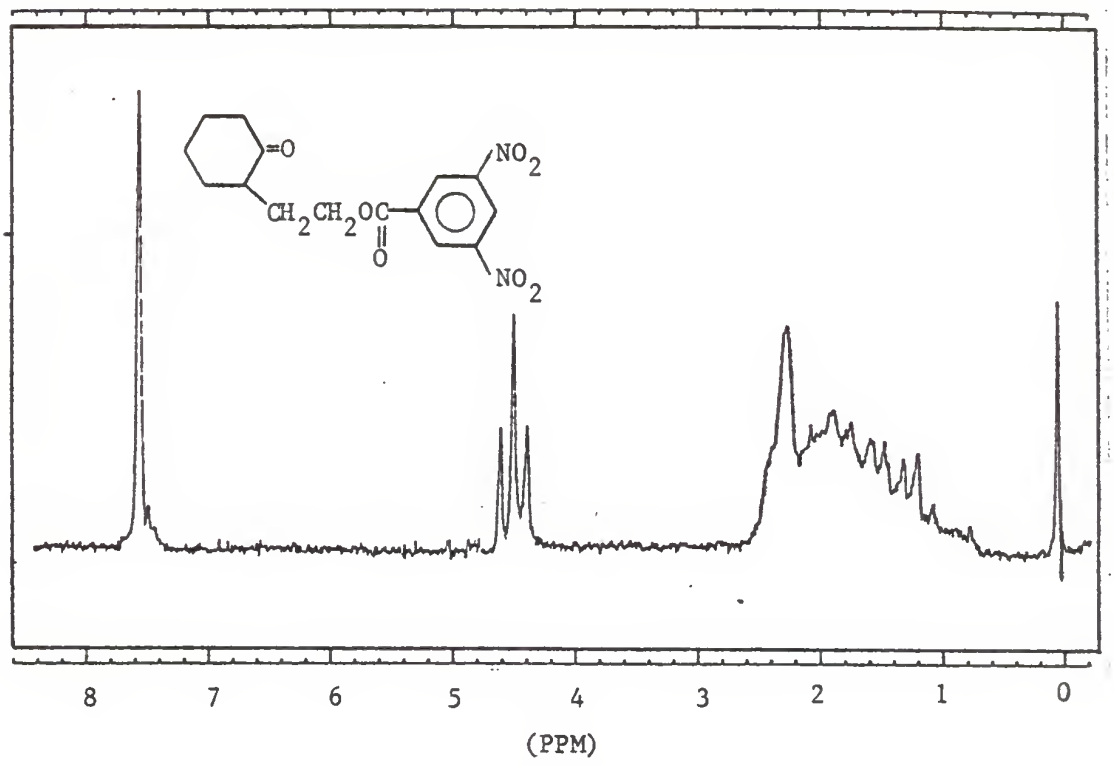
2-(2-Oxocyclohexyl)ethyl acetate

(CCl_4, Internal TMS)

2-(2-Oxocyclohexyl)ethyl 3,5-dinitrobenzoate

(CCl_4, Internal TMS)


=O
CH2CH2OCOCH3
8
7
6
5
4
3
2
1
0
(PPM)


=O
CH2CH2OC
O
NO2
NO2
8
7
6
5
4
3
2
1
0
(PPM)

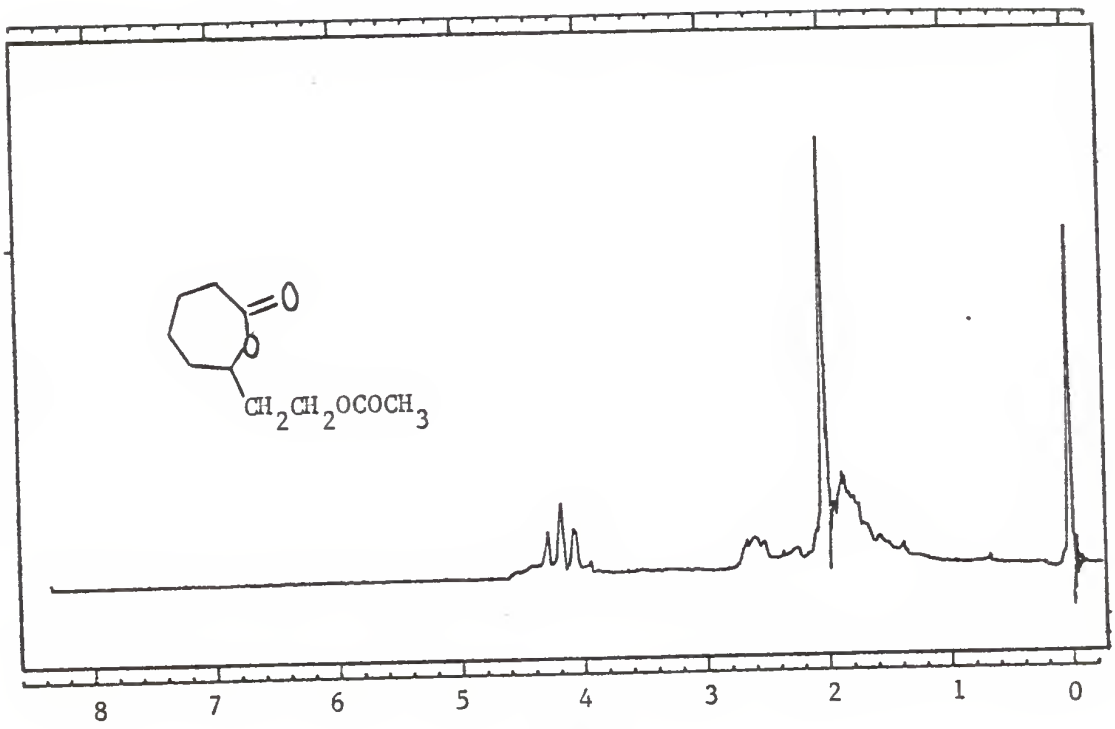
NMR SPECTRA

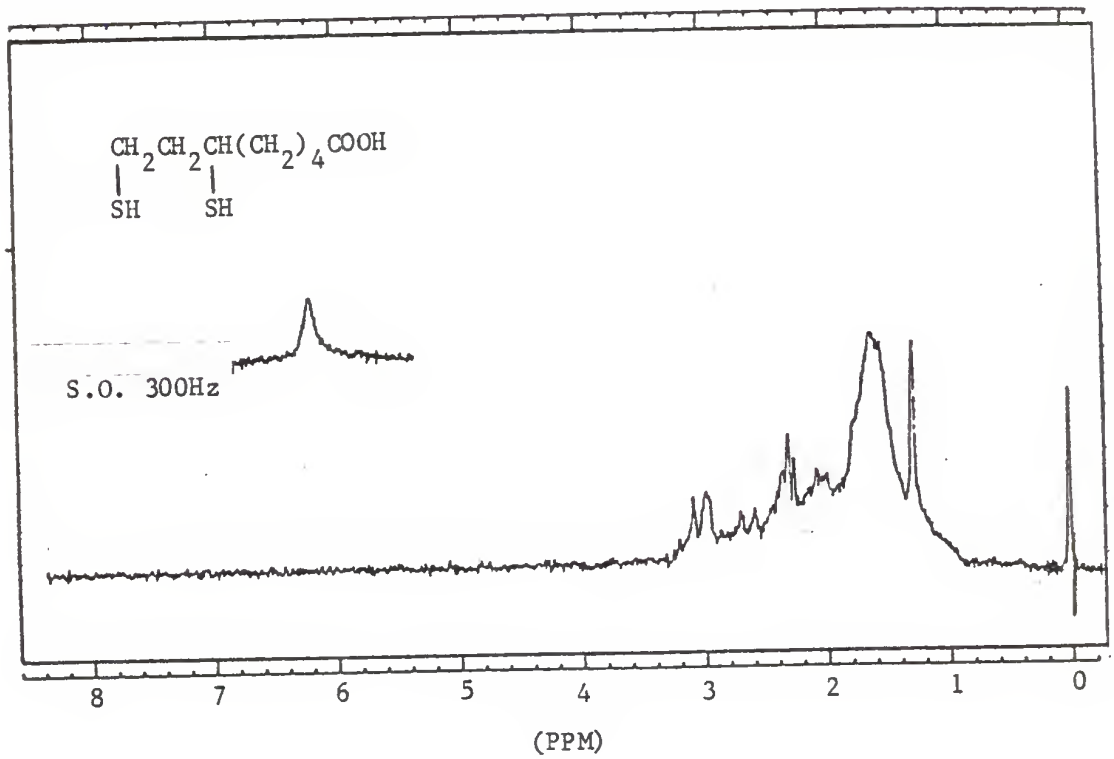
6-Hydroxy-8-acetoxyoctanoic acid 1—6-lactone

(CCl_4, Internal TMS)

Dihydrolipoic acid

(CCl_4, Internal TMS)


O
O
CH2CH2OCOCH3
8
7
6
5
4
3
2
1
0
(PPM)


CH2CH2CH(CH2)4COOH
SH
SH
S.O. 300Hz
8
7
6
5
4
3
2
1
0
(PPM)

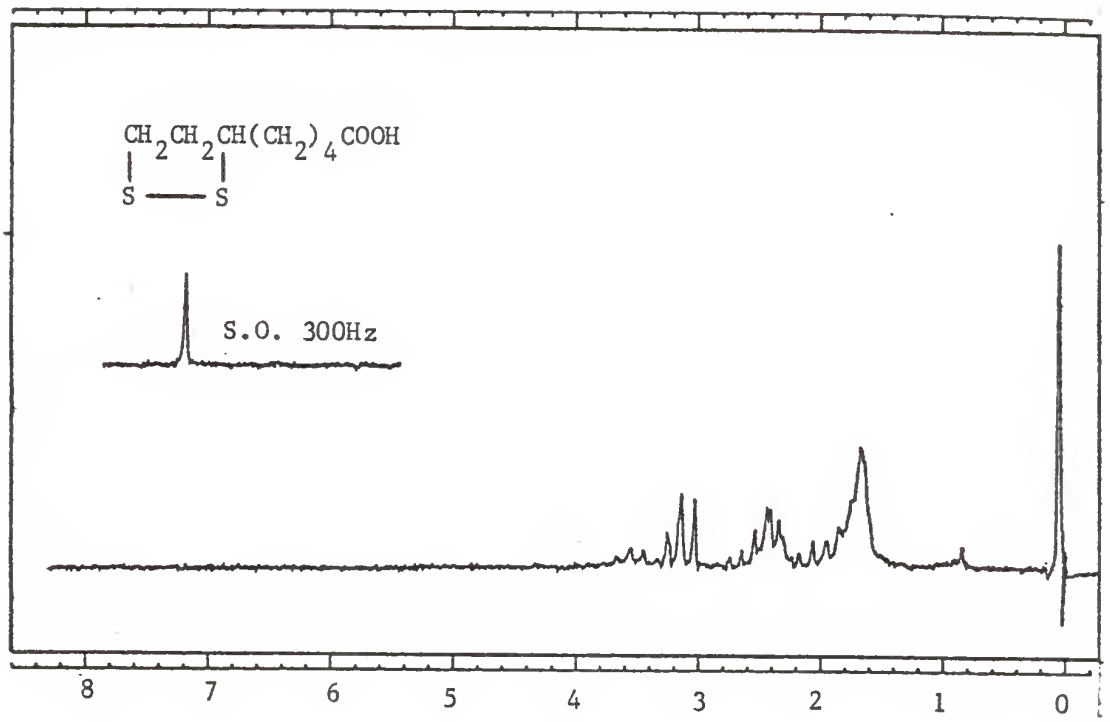
NMR SPECTRA

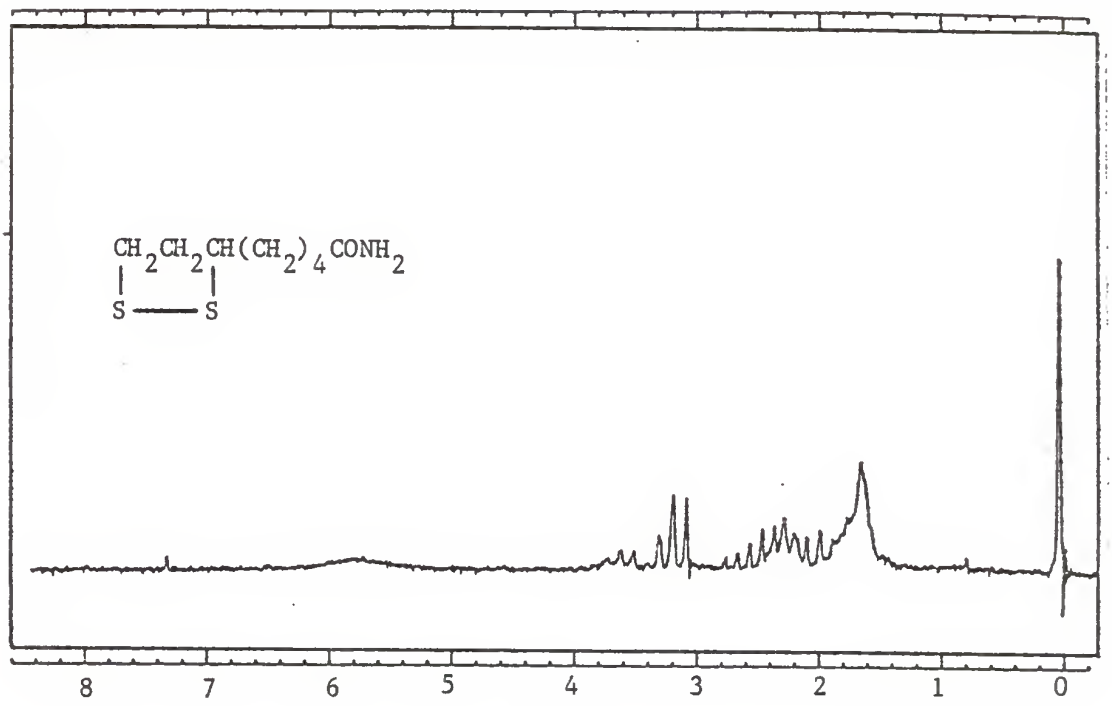
Lipoic acid

(CCl_4, Internal TMS)

Lipoamide

($CDCl_3$, Internal TMS)


CH2CH2CH(CH2)4COOH
S — S
S.O. 300Hz
8
7
6
5
4
3
2
1
0


CH2CH2CH(CH2)4CONH2
S — S
8
7
6
5
4
3
2
1
0

(PPM)

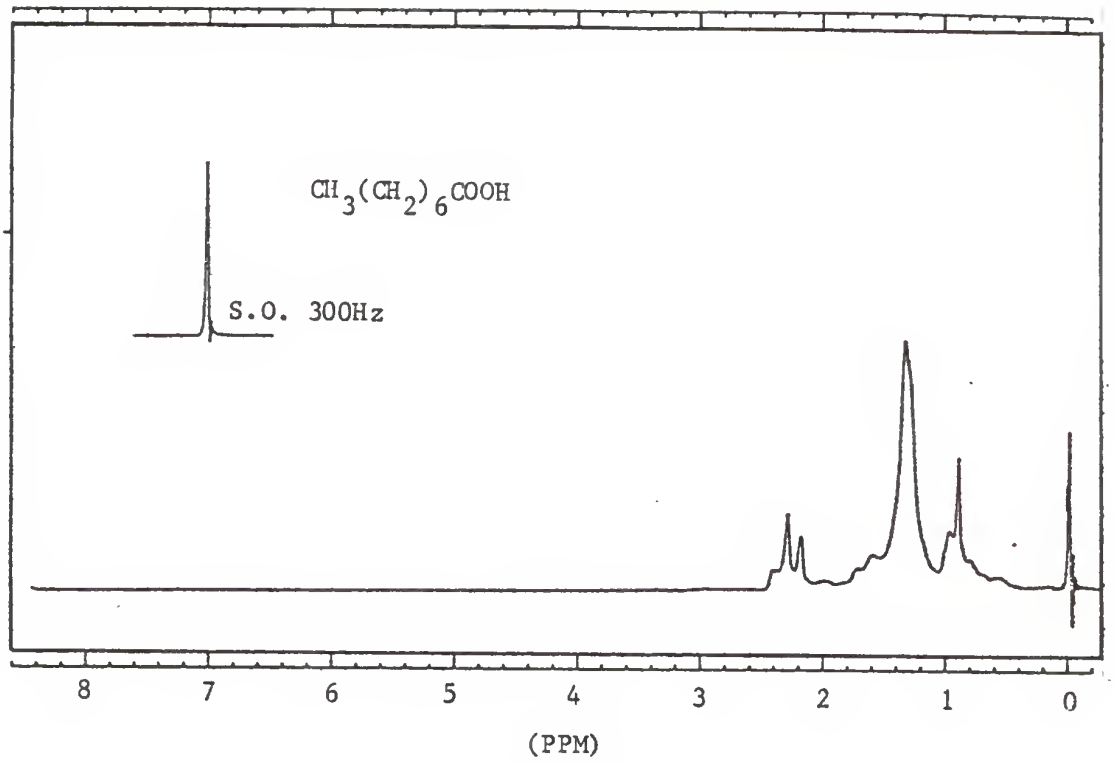
NMR SPECTRA

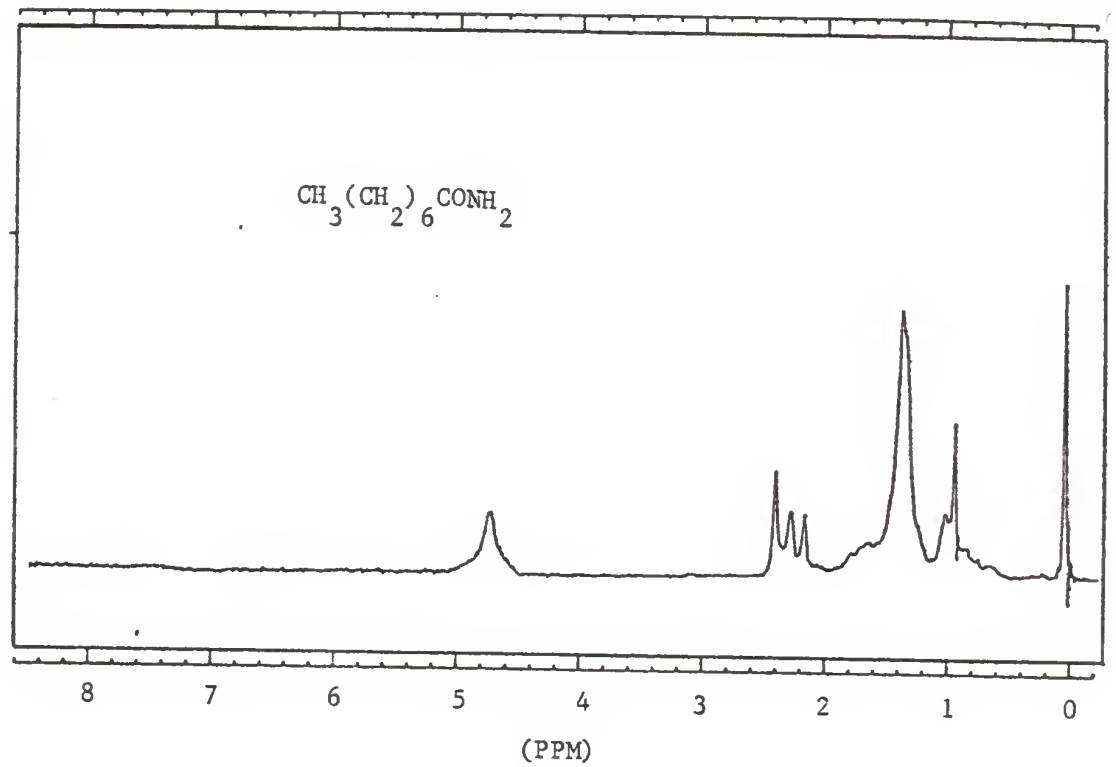
Octanoic acid

(CCl_4, Internal TMS)

Octanamide

(CCl_4, Internal TMS)


$CH_3(CH_2)_6COOH$
S.O. 300Hz
8
7
6
5
4
3
2
1
0
(PPM)


$CH_3(CH_2)_6CONH_2$
8
7
6
5
4
3
2
1
0
(PPM)

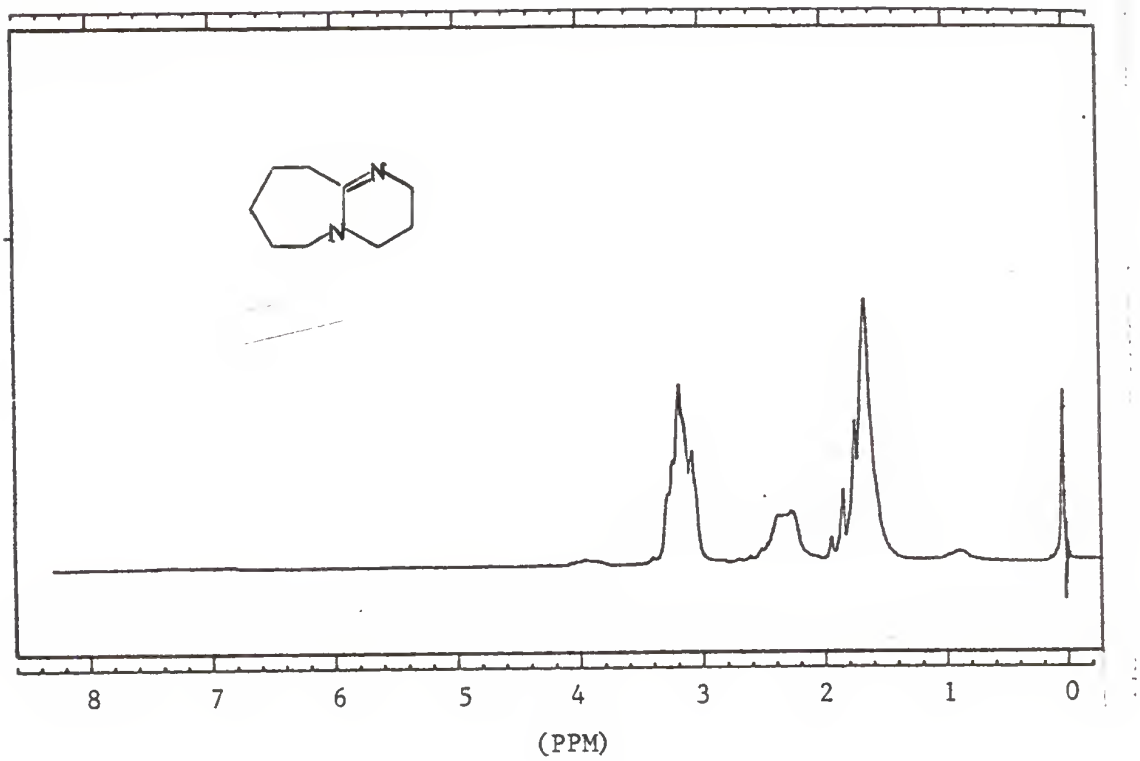
NMR SPECTRA

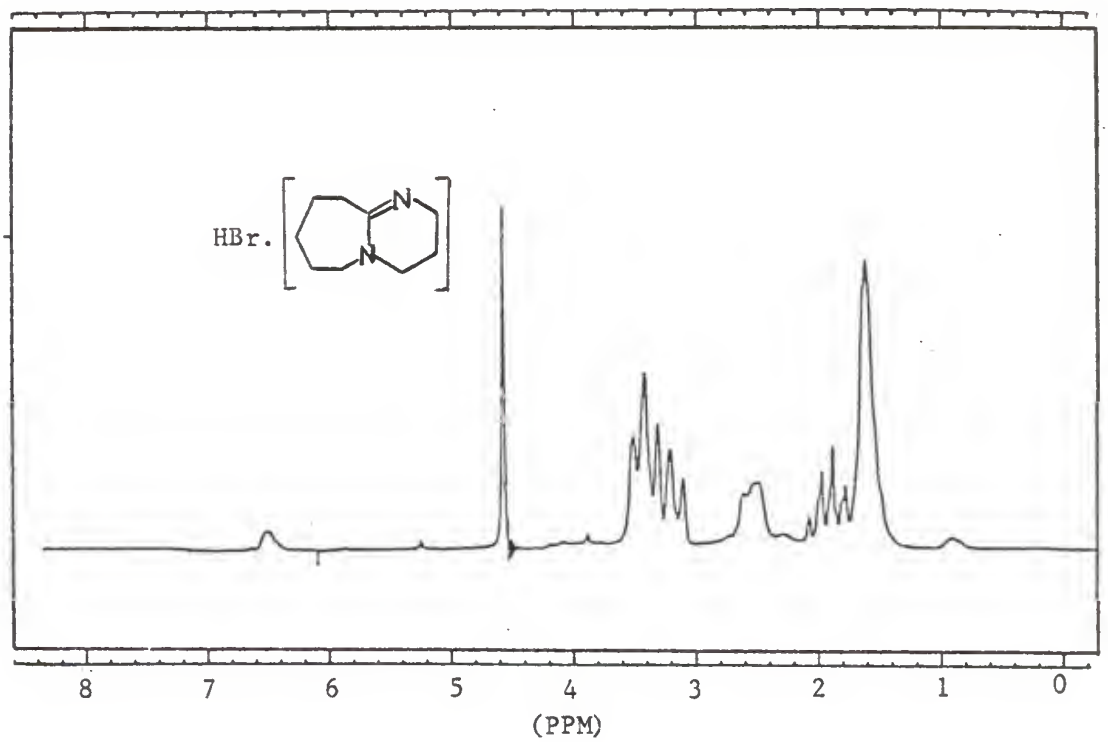
1,5-Diazabicyclo [5.4.0] undec-5-ene

(CCl_4, Internal TMS)

1,5-Diazabicyclo [5.4.0] undec-5-ene.hydrobromide

(D_2O)

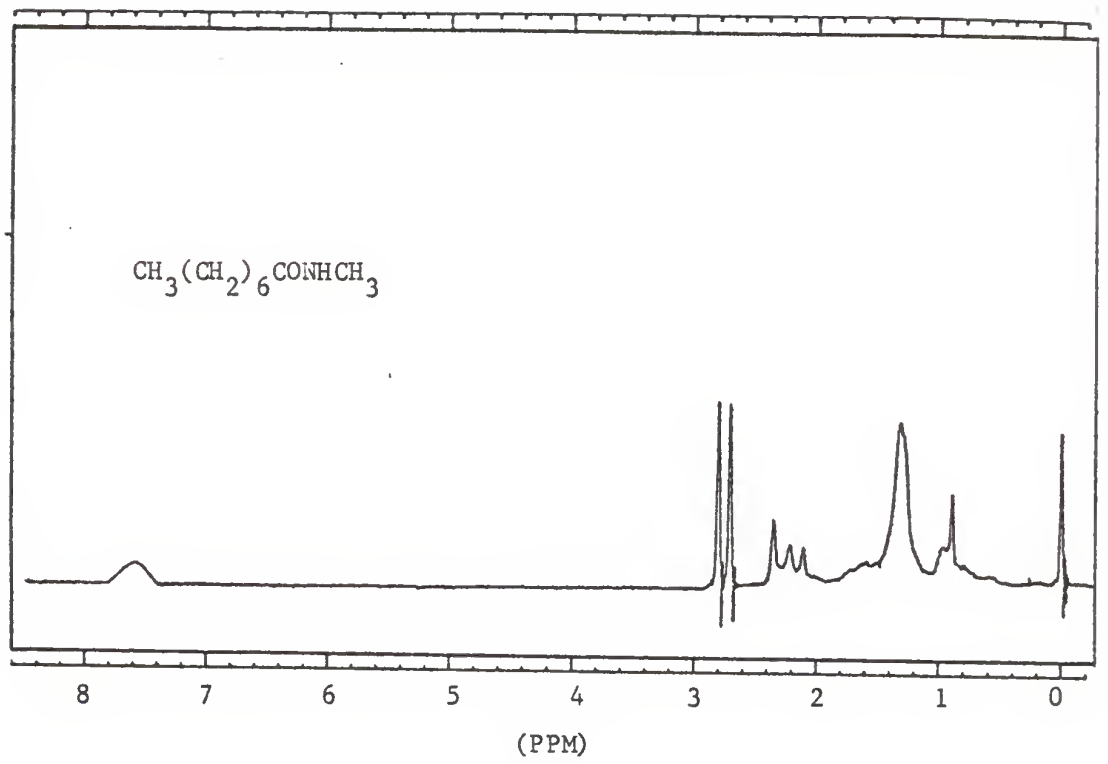

8
7
6
5
4
3
2
1
0
(PPM)


HBr.
8
7
6
5
4
3
2
1
0
(PPM)

NMR SPECTRA

N-Methyl octanamide

(CCl_4, Internal TMS)


CH3(CH2)6CONHCH3
8
7
6
5
4
3
2
1
0
(PPM)

A MORE CONVENIENT ROUTE TO LABELED LIPOIC ACID

by

KHUONG HOANG XUAN MAI

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfilment of the

requirements for the degree

MASTER OF SCIENCE

Department of Chemistry

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1979

ABSTRACT

Lipoic acid is an essential component of the multienzyme system which catalyzes the oxidative decarboxilation of pyruvic acid and other ∝-keto acids. It functions in acyl transfer to Coenzyme A and electron transfer to flavin adenine dinucleotide. The transferred acetyl group may be attached to lipoic acid at either of the two positions, 6 or 8. It may be possible to determine the exact position of attachment by using NMR or radioactive detection techniques to study the role of lipoic acid in the reactions catalyzed by the multienzyme complex.

This work furnishes a convenient route to labeled lipoic acid. With the labeled material it would be feasible to investigate the functions of lipoic acid. 1-Pyrrolidinocyclohexene was alkylated with ethyl bromoacetate to give the intermediate alkylated iminium bromide which was in turn converted into the alkylated enamine by a strong organic base. The enamine, ethyl (2-pyrrolidinocyclohexenyl)acetate, was reduced with lithium aluminum hydride, followed by hydrolysis in aqueous methanol to give 2-(2-hydroxyethyl)cyclohexanone. The latter was acetylated and peracid oxidized to 6-hydroxy-8-acetoxyoctanoic acid 1—6-lactone which was then reacted with thiourea and a strong mineral acid, followed by hydrolysis and oxidation to yield DL-lipoic acid.